Systematic Optimization of Bone Pharmaceuticals to Maximize Therapeutic Response in Conditions of Low Bone Mass

by

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Dedication

Para mi esposo, gracias por darme las alas para vivir una vida sin limites.

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Abstract

Osteogenesis imperfecta (OI) is a genetic disorder caused by collagen-related mutations which leads to increased bone fragility and low bone mass. Although the past decade has been marked by numerous advances in therapies that aim to stabilize the onset of metabolic bone disease, current treatment strategies leave room for substantial improvements. The studies that will be presented in this thesis focus on designing systematic treatments for two challenging clinical scenarios that require novel approaches. All studies have been approached in the context of OI using the Brtl/+ mouse model.

While the maternal skeleton goes through significant bone loss during pregnancy and lactation, this period of skeletal vulnerability can exacerbate an underlying metabolic bone condition like OI. In view of increasing use of bisphosphonates (BP) in premenopausal women to treat OI, the potential risks from long-term exposure on both maternal and neonatal skeleton during pregnancy and lactation remain inconclusive. When we assessed the maternal skeletal changes during pregnancy and lactation in Brtl/+ dams, pregnancy led to maternal trabecular gains in vertebral bone mass, while lactation induced maternal cortical and trabecular bone loss in both vertebra and femur. When BPs were administered prior to conception, bone mass gains due to pregnancy were amplified and lactation-induced bone loss was prevented. However, this protective effect was more modest with BP intervention during pregnancy, and ceased to exist in the late stages of lactation. Despite preventing lactation-induced maternal bone loss, no negative skeletal effects of BPs on offspring were observed. These findings indicate that during this period of

significant imbalance between bone resorption and formation, BPs can help reduce the risk of maternal bone fragility in OI by inhibiting lactation-induced bone resorption without affecting bone development in their offspring.

The second half of this thesis explores clinical cases with a critically depleted bone structure, such as severe OI. These cases pose a challenge to current antiresorptive and anabolic therapeutics since their response mechanisms target different abnormalities in the bone remodeling cycle. In this study, rapidly growing Brtl/+ mice were treated with a combination of pamidronate (PAM) and an anabolic (SclAb) in order to attain superior bone mass and strength effects compared to monotherapy. Results from this study showed that following one cycle of combination therapy, a single dose of PAM in combination with SclAb led to a cumulative effect on bone mass, but each through independent means. PAM retention mechanism led to an increase in trabecular number as the dosage increased while no additional gains were observed with SclAb. Conversely, while PAM showed no significant effect on trabecular thickness, SclAb induced a consistent trabecular thickening across all BP dosages. Chronic effects of concurrent administration of BP and SclAb revealed that accumulating cycles conferred synergistic gains in trabecular mass and vertebral stiffness, suggesting a distinct advantage of both therapies combined.

Given the lack of knowledge regarding the effects of BPs during reproductive periods and lack of treatment options for patients with severe OI, this thesis provides valuable insight that can help develop patient-specific treatment plans. By understanding the changes in bone metabolism of the clinical conditions we are trying to resolve, and by combining this knowledge with our understanding of the targeted pathways of available pharmaceuticals, we can strategically and systematically optimize bone therapeutics so that the best clinical outcome can be achieved.

Chapter 1 Introduction

Bone is a metabolically active tissue that undergoes continuous change through the process of bone resorption and bone formation [1]. This lifelong remodeling process, guided by osteoblasts and osteoclasts, is essential for maintaining optimal bone strength and mineral homeostasis [2]. Normally, remodeling is coupled such that the level of resorption is equal to the level of formation and bone density remains constant. In disorders of bone fragility, these functions become impaired and bone begins to deteriorate in composition, structure and functionality. The most prevalent example of such disorders is osteoporosis, which is marked by a decrease in bone mineral density (BMD) and strength. However, disorders of bone fragility may arise from a variety of different causes, with differing pathologies contributing to fragility. Such is the case of the rare metabolic bone disorder known as osteogenesis imperfecta which results from inherited genetic mutations and results in variable skeletal fragility over time.

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI), or brittle bone disease, is a genetic metabolic bone disorder caused by collagen-related mutations with an estimated prevalence of approximately 1 per 20,000 births [3]. Although symptoms vary widely among patients, the skeletal phenotype most commonly associated with OI is low bone mass, altered bone quality, and imbalanced bone remodeling [4]. As a result, bone fragility is a cardinal symptom in OI with its severity ranging from mild to severe.

Clinical observations have shown that the annual fracture rate in OI patients is most prominent during childhood in both males and females [5]. Though the symptoms vary over time, the condition persists throughout life. Dominant forms of OI encompass about 90% of clinical cases and are characterized by mutations in one of the two genes, COL1A1 and COL1A2, encoding the α1 and α2 chains of type I collagen [6]. The clinical severity from these mutations ranges from mild to perinatal lethal and currently more than 2500 distinct variants in the COL1A1 and COL1A2 genes have been identified [7]. For instance, individuals with a mild clinical diagnosis of OI, such as type I, have been shown to have one null allele from a premature translation termination codon which consequently reduces secretion of normal procollagen by 50% [8]. In more severe cases, the mutation is most often caused from an amino acid substitution for glycine, while few non-glycine substitutions in the triple helical domain have been reported [9-13]. In addition to these point mutations, splice site mutations can also result in the production of abnormal and impaired collagen type I molecules [6]. During collagen synthesis, two α1 chains and one α2 chain assemble into a triple helix structure where glycine, the smallest amino acid, is required at every third position of the peptide chain to produce a tightly packed and stabilized helical structure [14]. Therefore, point mutations of even one glycine lead to delayed folding of the triple helix that can cause asymmetric post-translational overmodification of the chains. On the other hand, splice site alterations can lead to in-frame sequence alterations or cause exon skipping [6]. The remaining clinical cases are recessive forms of OI caused by deficiency or mutations of genes encoding proteins required for collagen post-translational modifications, folding or secretion of type I collagen [4]. However, to date, it remains unclear how mutation type, location, and gene contribute directly to OI bone fragility.

In an effort to describe the range of phenotypic severity in OI, Sillence proposed a numerical classification scheme based on clinical and radiographic features [15]. Type I is a non-deforming form of OI that in spite of normal height may be expressed through blue-tinted sclera and a higher than normal fracture risk. More severe cases include perinatal lethal type II, progressive deforming type III and moderate deforming type IV. More recently, molecular genetics have identified more types of OI (up to type XVIII) [16], but types I-IV encompass the bulk of OI cases. In this thesis we will be focusing on OI type VI.

Therapeutics for Osteogenesis Imperfecta

Currently there is no cure for OI, but treatments have been designed with the goal to decrease pain, prevent fractures and improve mobility. Current treatment approaches for managing OI are comprised of physical therapy, rehabilitation, orthopaedic surgery and medical-pharmacological management. Although surgical interventions aim to prevent and correct long-bone deformities that impair function, due to the bone's poor quality, multiple surgeries are required for their success which may lead to further complications and increase time spent in the hospital. While these types of surgeries remain the mainstay of treatment in children with OI, advances in the pharmacological management of OI have focused on stabilizing the underlying metabolic disease by addressing its low bone mass characteristics[17].

At the cellular level, patients with OI have increased osteoclastic bone resorption, thus bisphosphonates (BP) are considered the standard care for treating OI due to their ability to inhibit osteoclast activity[18]. BPs have been known to chemists since the middle of 19th century, when they were mainly used as corrosion inhibitors or as agents in textile, fertilizer, and oil industries.

However it wasn't until observations of BPs ability to inhibit the dissolution of hydroxyapatite crystals that led to their use as therapeutic interventions for metabolic bone diseases [19]. Various OI clinical studies have shown BPs effectiveness in decreasing bone turnover and increasing bone mineral density [20-26]. However, it is important to note that BPs do not actively build bone tissue. Rather than directly addressing osteoblasts that harbor the OI-causing collagen mutation, their effect is strictly on inhibiting bone resorption. As a result, BPs effect on OI fracture prevention remains inconclusive. Studies using an OI mouse model with a phenotype of spontaneous fractures showed that alendronate treatment increased bone volume and decreased fracture incidence [27, 28]. However in another OI animal study using antiresorptive denosumab, an antibody against RANKL, a significant increase in bone mineral density was observed but fracture incidence was not reduced [29]. Similar observations have been shown in clinical studies where fracture incidence was reduced in the vertebral body but not in long-bones [21, 24, 30-33].

Recently, therapies with bone-anabolic capabilities have attracted general interest, especially since genetic defects underlying OI primarily affect collagen type I which is synthesized by osteoblasts. A study using growth hormone therapy showed the drug's effectiveness in increasing linear bone growth, but its effects were not seen in all pediatric patients [34]. Significant increase in bone density with teriparatide, a recombinant human form of parathyroid hormone, was observed in adults with mild OI [35]. However, currently the FDA has not approved its used in children since preclinical rat studies showed the potential risk of osteosarcoma induction in addition to increased bone mass [36]. Sclerostin is an antagonist to the Wnt signaling pathway which has increasingly received attention as a potential target for anabolic treatment of metabolic bone diseases. Sclerostin is encoded by the SOST gene and is primarily expressed in mature osteocytes [37]. The role of sclerostin in bone development was identified when studies showed that bone overgrowth

disorders like sclerosteosis and van Buchem disease were caused by inactivating mutations in the SOST gene [38, 39]. In both animal models and case studies, loss of sclerostin activity has shown an increase in osteoblast activity and an upregulation in bone formation markers which consequently induces a significant increase in bone mass [40, 41]. Conversely, transgenic mice overexpressing the SOST gene exhibited osteopenia due to inhibited osteogenic differentiation and stimulated osteoblast apoptosis [42, 43]. As a result, monoclonal inhibiting antibodies that mimic the loss of sclerostin/SOST were developed and initially tested in animal models of osteoporosis. These preclinical studies confirmed the anabolic efficacy of sclerostin antibody (SclAb) where a significant increase in bone mass and strength was observed [44-46]. Due to sclerostin's potency in inhibiting bone formation in cases of osteoporosis, its application in OI has recently been explored. Our group and others have invested considerable effort into establishing the anabolic efficacy of SclAb in a variety of animal models with an OI phenotype [47-53].

Thesis Motivation

Although the past decade has been marked by numerous advances in therapies that aim to stabilize the onset of metabolic bone disease, current treatment strategies leave room for substantial improvements. In this thesis, we have focused on two challenging clinical scenarios that require novel therapeutic approaches:

1) Pregnancy and lactation-associated osteoporosis (PLO) and pregnancy associated osteoporosis (PAO) are rare form of osteoporosis characterized by the occurrence of fracture during late pregnancy or during the postpartum period [54, 55]. While the maternal skeleton goes through significant bone loss during pregnancy and lactation, this period of skeletal vulnerability has

not been clearly associated with increased maternal fracture risk. However, these physiological conditions can exacerbate an underlying metabolic bone condition like OI [56, 57]. In view of the increasing use of BPs in premenopausal women to treat OI, the potential risks from long-term exposure on both maternal and neonatal skeleton during pregnancy and lactation remain inconclusive. Even if administration has been ceased during pregnancy, due to their long half-life, there remains a risk that embedded BPs can be released into the bloodstream and consequently cross the placenta or diffuse into the breast milk during these periods of high calcium demand. Additionally, since BPs have been known to suppress osteoclastic resorption, their effect on pregnancy and lactation-induced bone loss can lead to changes in calcium homeostasis and indirectly affect fetal and neonatal bone development. We hypothesize that BP intervention during pregnancy and lactation will induce a temporal effect on trabecular and cortical maternal bone structure that is representative of the changes in calcium metabolism that are occurring during these reproductive periods. Identifying this therapeutic window can help minimize this period of maternal skeletal vulnerability that can be exacerbated in women with an underlying disease like OI.

2) Clinical cases with a critically depleted bone structure, such as severe osteoporosis and severe OI, pose a challenge to current antiresorptive and anabolic therapeutics since their response mechanism target different abnormalities in the bone remodeling cycle. Although both antiresorptive and anabolic agents have clinically shown significant improvements in bone mineral density [25, 58, 59], they have both failed to achieve an adequate response in cases where trabecular structure is limited due to excessive bone turnover [60-63]. Since antiresorptive agents preserve trabecular number by targeting osteoclasts and anabolic agents induce trabecular thickening by targeting pathways of osteoblast-mediated bone formation,

combining both therapeutics may provide distinct advantages over either treatment alone. We hypothesize that a minimal BP dose, that would otherwise be too weak to preserve long-term bone mass, will preserve primary trabecular architecture and serve as a substrate for additional bone formation with subsequent SclAb treatment.

Thesis Overview

The studies that will be presented in this thesis have been approached in the context of OI using the Brtl/+ mouse model. This knock-in model for moderately severe Type IV OI is heterozygous for a glycine to cysteine (G349C) substitution in one COL1A1 allele, reproducing features observed in a clinical phenotype such as low bone mass and skeletal fragility [64]. The Brtl/+ mouse have been proven suitable for studying the effects of therapeutics in OI since it recapitulates the clinically observed phenotype [65-67].

Chapter 2: Bisphosphonate Treatment Intervention during Pregnancy and Lactation Leads to Temporal Inhibition of Lactation-Induced Bone Loss on the Maternal Skeleton

Chapter 2 addresses the significant loss in maternal bone structure that may exacerbate metabolic bone disorders like OI and its effects when treated with BPs. While pregnancy and lactation-induced bone loss is not clearly associated with increased maternal fracture risk, these physiologic conditions still pose several potential challenges in women with OI. It is not recommended to use pharmaceuticals during pregnancy and lactation, however, OI patients are more likely to be treated with BPs prior to conception than any other population. In fact, case studies have reported instances where BP therapy is stopped as soon as pregnancy is confirmed in women with OI [68]. Upon administration, BPs initially bind to hydroxyapatite crystals within the bone matrix and

become sequestered in the bone matrix [18]. Activation of BPs occurs when they are released from the bone surface by osteoclasts, which subsequently reduces bone turnover through inhibition of osteoclast activity [69]. Because BP can remain embedded within the bone matrix until they are taken up by osteoclasts, there is rising concern that premenopausal women placed on BP early in life might encounter drug release during pregnancy or lactation, resulting in fetal or neonatal exposure with unknown consequences.

During pregnancy, the placenta plays a crucial role in nourishing the fetus by enabling the transfer of oxygen, carbon dioxide and essential vitamins and minerals from the maternal blood supply. For a long time, the general opinion was that the placenta served as a barrier that protected the fetus from harmful agents during pregnancy. However, it wasn't until the thalidomide tragedy, when numerous report of malformations in babies brought about awareness of the imperfect state of the placenta as a barrier to drug transfer [70]. Initially, studies focused on noticeable effects like congenital malformations and developmental disorders, but later it became clear that manifestation of teratogenicity also included infertility, spontaneous abortions, intrauterine death, premature birth, low birth weight, and organ failure; since exposure to certain drugs during pregnancy may alter the function of the placenta or interfere with the environment within the uterus [71, 72]. Similarly, during lactation, the physiologic process of breastfeeding plays an essential nutritional and protective role during infant development [73]. However, since it is well established that anything taken by the nursing mother may diffuse into the breast milk [74-77] and therefore become bioavailable to the infant, it has also become imperative to establish the potential risks in neonatal development following drug exposure.

Although the majority of unbound medication is transported across the placenta by passive diffusion, several other factors (protein binding, pH difference or lipid solubility) can affect their

ability to cross the placenta [78]. Pharmaceuticals with a low molecular weight (< 500 g/mol) diffuse freely across the placenta, those with moderate molecular weights (500 – 1000 g/mol) cross the placenta less easily, while those with a high molecular weight (> 1000 g/mol) do not cross the placenta [78]. As a result, several studies have shown that BP, with a molecular weight of between 235.07 g/mol and 249.096 g/mol, crosses the placenta in various animal models [79-82]. However, these studies have failed to agree on the drug's effects on the developing fetus after *in utero* exposure. Moreover, human reports concerning women exposed to BPs before conception or during pregnancy did not demonstrate serious adverse effects to fetuses [83-87].

The transfer of drugs into the breast milk shares some of the same principles as crossing the placenta, mostly through passive diffusion. Studies have shown that medication may diffuse into the milk and back into the bloodstream based on the amount of drug concentration in maternal serum [88]. Similar to crossing the placenta, molecular weight can predict a medication's likelihood to cross into breast milk. Medications with small molecular weights (< 200 Da) are more likely to cross than those with a larger molecular weight. However, many other properties may also affect their diffusivity such as bio-availability, maternal plasma levels, pH, and lipid solubility [88]. To date, there are three animal studies that explore the effects of BPs during lactation, however similar to studies assessing BP safety during pregnancy, recommendations regarding the use of BP during lactation remain inconclusive [89-91]. With regards to human data, there is a dearth of case studies reporting the effects of BPs prior to or during lactation. Of the case studies that are available, both mother and infant did not demonstrate serious adverse effects [68-70]. Based on the data available in the literature, there remains considerable debate about the effects of BPs on the maternal, fetal, and neonatal skeleton, and how these affects may change with timing of exposure. To assess this clinical scenario, Brtl/+ females were used to identify the

maternal skeletal changes during pregnancy and lactation in OI and how these skeletal changes were affected with PAM intervention. We also explored how treating the maternal skeleton with PAM during pregnancy and lactation might affect fetal and neonatal skeleton development. Four possible routes of fetal and neonatal PAM exposure that might alter bone metabolism were considered: 1) PAM can cross the placenta if the mother is treated during pregnancy. 2) PAM can diffuse into the breast milk if the mother is treated during lactation. 3) Because of BPs long half-life, treating the maternal skeleton prior to conception may expose fetal and neonatal bone to PAM through their recycling secondary to maternal bone resorption. 4) PAM can indirectly interfere with fetal and neonatal development by inhibiting maternal bone resorption. This in return can disrupt calcium homeostasis and prevent offspring from receiving the necessary calcium supply for healthy bone development.

Our results showed that pregnancy led to maternal trabecular gains in vertebral bone mass, while lactation induced maternal cortical and trabecular bone loss in both vertebra and femur. When the maternal skeleton was treated with PAM prior to conception, bone mass gains due to pregnancy were amplified and lactation-induced bone loss was prevented. This protective effect was more modest when given during pregnancy (E15) and ceased to exist when PAM was given during the late stages of lactation. Furthermore, pregnancy induced osteocyte osteolysis which was unaffected with PAM treatment. Despite preventing lactation-induced maternal bone loss, no negative skeletal effects of BPs on offspring were observed. These findings indicate that during this period of significant imbalance between bone resorption and formation, BPs can help reduced the risk of maternal bone fragility in OI by inhibiting lactation-induced bone resorption without affecting bone development in their offspring.

Chapter 3: Low Dose of Bisphosphonate Enhances Sclerostin Antibody-Induced Trabecular Bone Mass Gains in Brtl/+ Osteogenesis Imperfecta Mouse Model

Chapter 3 addresses the poor bone response from antiresorptive and anabolic monotherapy in cases associated with severely low bone mass. Antiresorptive and anabolic monotherapy as described earlier, have shown a beneficial response in bone mass in both animal models and clinical studies. However, the bone mass gains produced from antiresorptive therapies like BPs do not always yield a decrease in fracture incidence. While SclAb has gained interest as a promising anabolic approach for the treatment of OI, in severe cases of OI, less promising results were observed [60]. Thus, we have proposed a combination of antiresorptive and anabolic treatments for these challenging clinical cases.

The rationale for combining anabolic and antiresorptive agents is that if bone formation is stimulated by an anabolic agent while bone resorption is inhibited by an antiresorptive agent, the concomitant combination therapy might attain superior bone mass and strength effects compared to monotherapy. Clinical studies have observed the effects of anabolic and antiresorptive treatments and demonstrated their superiority over monotherapy in improving BMD [92-94]. However, there is a clear difference in the data found between the order, timing and dosage of treatment. In an OI mouse model, BP treatment helped preserve SclAb-attributed bone mass gains following cessation of drug [53]. Alternatively, when SclAb was administered following BP in aged ovariectomized rats, gains in bone mass with SclAb were unaffected by prior BP exposure [45]. However, in another OI mouse model, when SclAb was combined with zoledronic acid a saturation effect was observed from zoledronate treatment that no additional gains in bone mass were observed with SclAb [95].

In this study, rapidly growing Brtl/+ mice were treated with PAM and SclAb to assess the immediate and long-term effects of combination therapy in a model of moderately-severe OI. In order to minimize the effects of PAM, we chose dosages that represent 10% (0.3 mg/kg) and 20% (0.625 mg/kg) of dosage shown to induce trabecular retention in growing mice [96]. Sclerostin antibody was chosen at a dose that consistently induced an anabolic effect in our prior studies in the Brtl mouse [47, 48]. Our goal in designing this study was to allow both drugs to work together rather than creating a saturation effect from one of the drugs.

Results from this study showed that following one cycle of combination therapy, a single dose of antiresorptive PAM in combination with anabolic SclAb led to a cumulative effect on bone mass, but each through independent means. PAM retention mechanism led to an increase in trabecular number as the dosage increased while no additional gains in number were observed with SclAb. Conversely, while PAM showed no significant effect on trabecular thickness, SclAb induced a consistent trabecular thickening across all BP dosages. Chronic effects of concurrent administration of BP and SclAb revealed that accumulating cycles conferred synergistic gains in trabecular mass and vertebral stiffness, suggesting a distinct advantage of both therapies combined. Cortical gains in mass and strength occurred through SclAb alone, independent of presence of BP. In conclusion, these preclinical results support the scientific hypothesis that a single dose of antiresorptive BP facilitated the anabolic action of SclAb by increasing availability of surfaces for new bone formation via retention of primary trabeculae that would otherwise be remodeled.

Thesis Contributions

Given the lack of treatment options for patients with severe OI and the lack of knowledge regarding the effects of BPs during pregnancy and lactation on both the maternal and fetal/neonatal skeleton, this thesis provides valuable insight that can help develop patient-specific treatment plans to optimize the effects of current and promising therapies for both premenopausal women and pediatric OI. To date, various pharmaceutical interventions have been studied extensively in the pediatric population since bone fragility is high prior to achieving peak bone mass. The aging population has also been extensively evaluated with antiresorptive and anabolic treatments due to increased risk for developing osteoporosis. Although studies have shown the benefits in treating young adults, post-puberty, bone fragility in OI is relatively low during this time.

Little is known about bone fragility in premenopausal women who become pregnant and lactate their offspring. It is known that the maternal skeleton experiences significant bone loss during pregnancy and lactation in order to provide the necessary calcium supply to their developing offspring. Although significant bone mass gains have been observed months after lactation, this period of skeletal vulnerability can increase fracture risk especially in women associated with an underlying metabolic bone disorder like OI. In Chapter 2, we addressed this clinical challenge by treating Brtl/+ mice with a single clinical dose of PAM at one timepoint during pregnancy and lactation. The resulting temporal effects on bone metabolism from a single BP dose administered during pregnancy or lactation provide a treatment window for when BPs are most effective on the maternal skeleton with no adverse effects on fetal/neonatal bone development. Thus, in cases where the maternal skeleton can benefit from BP intervention such as pregnancy and lactation-associated osteoporosis (PLO), glucocorticoid-induced osteoporosis, or hypercalcemia, this

optimization window can provide insight on time points of intervention that can yield beneficial clinical outcomes.

Another gap in the OI bone fragility timeline is the lack of promising treatment options for children with severe OI. Although a variety of studies have evaluated the effects of antiresorptive and anabolic monotherapy in pediatric OI, no single therapy has led to significant improvements in severe OI. In Chapter 3 we addressed this clinical challenge by treating rapidly growing Brtl/+ mice with a carefully designed PAM and SclAb combination therapy. The synergistic effects observed from this study provide a potential treatment plan for patients that lack structural bone surface that will not benefit from monotherapy alone. By taking advantage of the antiresorptive effects of PAM and the anabolic effects of SclAb, we can prevent the conversion of primary to secondary spongiosa in a growing model so that SclAb can have enough surface to build on.

Through the presented work in this thesis I hope to further the field into developing more strategic and thought-out therapeutic interventions. Rather than developing new pharmacological drugs to achieve a "one treatment fits all" solution, we, the community, can design novel and personalized treatment interventions, especially for challenging clinical scenarios with an underlying metabolic bone disease. By understanding the changes in bone metabolism of the clinical condition under investigation and combining it with our understanding of the targeted pathways of antiresorptive and anabolic therapies, we can strategically and systematically optimize bone therapeutics so that the best clinical outcome can be achieved.

Chapter 2 Bisphosphonate Treatment Intervention during Pregnancy and Lactation Leads to Temporal Inhibition of Lactation-Induced Bone Loss on the Maternal Skeleton

Introduction

Pregnancy and lactation are known as periods of significant maternal bone loss due to changes in calcium homeostasis required for fetal bone mineralization and breast milk production. Since the decrease of maternal bone has been shown to be transitory, this reproductive period has not been clearly associated with osteoporosis or increased maternal fracture risk [97]. However, it remains uncertain if these physiologic conditions define a period of vulnerability for the maternal skeleton associated with a rare underlying metabolic bone disorder like osteogenesis imperfecta (OI).

Limited information on the maternal skeletal outcome following pregnancy and lactation in women with OI is known [98-100], however fractures and other complications have been reported [56, 101-103]. Currently, bisphosphonates (BPs) are the most widely prescribed anti-resorptive for the treatment of metabolic bone diseases associated with excessive bone resorption [18, 104]. Through their high affinity for calcium ions, these synthetic analogues bind to hydroxyapatite crystals within the bone matrix to mediate an inhibitory effect on active osteoclasts to decrease bone turnover and increase bone mineral density [20-26]. Although the majority of patients treated with BPs are postmenopausal, children and women of childbearing age are increasingly being prescribed these treatments [105-107]. Because BPs can remain sequestered within the bone matrix until they are taken up by osteoclasts, there is raising concern that fetal exposure could occur in cases where the mother has been subjected to BP treatment prior to conception. Additionally, it

has been shown that disease-modifying medication has the potential to pass from the mother's blood stream into the breast milk and reach the nursing infant [74-77]. Although clinical case studies have reported the benefits of BPs for the treatment of pregnancy and lactation-associated osteoporosis (PLO), glucocorticoid- induced osteoporosis, hypercalcemia and other complications during pregnancy and lactation [106, 108-111], data on the effects of BP exposure during pregnancy or lactation remains limited.

Currently BPs have been classified by the FDA as holding a category C pregnancy risk because animal reproduction studies have shown adverse effects, and no adequate and well-controlled studies in humans have been reported [112]. Our current knowledge about the effect of BPs on pregnancy is based almost entirely on animal studies, which have shown that these drugs are capable of crossing the placenta, thus putting the fetus at risk for exposure *in utero* [79-82]. Although these animal studies showed that BP exposure led to low birth weight, underdevelopment, and skeletal retardation, toxic levels were administered in order to assess their safety during pregnancy and lactation. Two toxicity animal studies have also explored the effects of BPs during lactation where severe and mild hypocalcaemia was observed due to disruption in maternal calcium metabolism [89, 113-115]. Of the case studies that are available, both mother and infant did not demonstrate serious adverse effects, however a few studies did report low birth weight, low gestational age and transient asymptomatic hypocalcaemia in the offspring [83-87, 116-119].

Recommendations regarding the use of BPs during lactation are often conflicting since it is not entirely known how much of a given drug passes the placenta or diffuses into breast milk and studies don’t agree on a common conclusion. Thus, there remains a need for developing systematic studies on BP effect when treating the maternal skeleton during a period of demanding metabolic

bone changes that are critical for the skeletal development of their offspring. In the present study we sought to determine a therapeutic window for when BPs are most potent on the maternal skeleton and how exposure affects fetal and neonatal development. We hypothesized that BP resorption effects on the maternal skeleton will be highly dependent on the time of administration since bone metabolism is constantly changing throughout pregnancy and lactation. Offspring are at risk of BP exposure if BPs cross the placenta or diffuse into the breast milk even if the mother has stopped treatment prior to conception, due to BPs long-half-life and their release into the bloodstream following maternal bone resorption. Additionally, since the maternal skeleton serves as a calcium reservoir to maintain calcium homeostasis during ossification and mineralization of their offspring, BPs can interfere with maintaining this balance and indirectly affect fetal/neonatal bone development. To explore these questions, we tested BP effects using a single injection of pamidronate at different stages of pregnancy and lactation in Brtl/+ dams harboring an OI-causing defect. Our knock-in mouse model reproduces the phenotype of moderately severe type IV OI [64-66] and has been used to explore therapeutic efficacy of anti-resorptive and anabolic interventions in growing and aged mice [47, 48, 50, 53, 120, 121]. Additionally, to explore the sequela of events induced by BP on the maternal skeleton, we analyzed fetal and neonatal bone development in genotyped and sexed offspring from treated and untreated Brtl/+ dams. In the present study, a temporal effect on the preservation of bone mass was observed when BP was administered at different stages of pregnancy and lactation. When BP treatment was administered prior to conception, lactation-induced maternal bone loss was prevented while BP-induced skeletal preservation was less effective when administered during the late stages of lactation. Despite preventing lactation-induced bone loss, no adverse effect on fetal and neonatal bone development

was found, suggesting additional sources of calcium are capable of responding to the demands placed on the maternal skeletal.

Materials and Methods

Experimental design

Brtl/+ mice with a mixed background of SV129/CD-1/C57BL/6S were derived from heterozygous Brtl/+ and WT parental strains [64]. To identify temporal maternal skeletal changes during pregnancy and lactation in OI and how these skeletal changes are affected by BPs, female Brtl/+ (n = 10/group) mice between 12 and 18 week of age were randomly assigned to receive a single tail vein injection of either pamidronate (PAM 3 mg/kg) or vehicle (PBS) during one of five time points (prior to conception (PC), gestation day 15 (E15), lactation day 1 (D1), lactation day 10 (D10) or lactation day 15 (D15)). This single PAM dose represents a standard clinical dosage that has been used during pregnancy [106]. For successful timed pregnancies, female Brtl/+ were mated with experienced male WT breeders and pregnancy was assessed by examining for a vaginal plug. Pregnant mice were caged individually and were continued on standard chow diet (5L0D, PicoLab, St. Louis, MO, USA) throughout pregnancy and lactation. Standard chow contained a calcium concentration of 6.6% and 4.6 IU/gm of vitamin D. Mothers, along with litter, were euthanized at birth to isolate the effects of pregnancy. To assess both pregnancy and lactation effects, mother and litter were euthanized at weaning (D21). Age- matched virgin female Brtl/+ mice (n = 10) were assessed to establish a skeletal baseline uninfluenced by pregnancy and lactation. A summary of the experimental design for maternal and neonatal assessment of skeletal changes during pregnancy and lactation under the influence of BPs is shown in Figure 2.1. All protocols and

procedures were approved by the University of Michigan's Committee on Use and Care of Animals.

Non-therapeutic fluorescent bisphosphonate probe

A single injection of commercially available far-red fluorescent pamidronate (FRFP) Osteosense-680EX (Perkin Elmer, Bedford, MA, USA) was administered in Brtl/+ pregnant (gestation day 19) and lactating dams (lactation day 1) to assess if BPs crossed the placenta and diffused into the mammary glands. The administered dosage was 2nmols of FRFP buffered in PBS in a tail vein injection volume of 150 µL. Controls received a 150 µL tail vein injection of PBS.

FRFP Imaging

To assess FRFP binding in fetal skeleton, pregnant Brtl/+ dams (gestation day 19) were euthanized within 24 hours of FRFP injection. Embryos were fresh frozen in chilled isopentane at −70°C in O.C.T compound embedding medium (Fisher Chemical Co., Santa Clara, CA, USA) and stored at -80°C. To assess if BPs diffuse into the breast milk during lactation, the mammary glands from Brtl/+ dams at day 1 of lactation were fresh frozen and stored similar to the embryos. Frozen samples were sectioned to a thickness of 10µm using the Kawamoto film technique [122] and a Leica CM3050S cryostat (Leica Microsystems, Germany). Fluorescent images from tissue sections were acquired with a 20x dry objective using a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Germany). To assess diffusion into breastmilk a similar protocol was carried out. However, prior to euthanizing Brtl/+ dams who were in lactation, dams were imaged in vivo with the Li-COR Pearl Animal Imaging.

Micro-computed tomography (μCT)

To analyze maternal and neonatal bone morphological changes after pregnancy and lactation, excised L3 vertebrae and right femora were first fixed in 10% neutral buffered formalin. Following fixation, both vertebrae and femurs were scanned in water using high-resolution μCT (Brucker Skyscan 1176, Kontich, Belgium). Image acquisition was performed at 50 kV and 800 μA with a 0.3° rotation step and a 0.25 mm aluminum filter for filtration of beam hardening artifacts. Manufacturer-provided hydroxyapatite phantoms were scanned with the same parameters to calibrate and compute volumetric BMD. Individual two-dimensional cross-sectional images were reconstructed into three-dimensional volumes with 9 μm isotropic voxel size using NRecon software (version 1.6.5.8, Bruker).

Two volumes of interest were established to analyze the effect of BPs during pregnancy and lactation on the femur. A 2 mm (maternal) and 1 mm (neonatal) VOI was centered midway between the lateral third trochanter and the distal femoral growth plate for cortical analysis and segmented using a user-defined global threshold derived from calculating the average thresholds over a range of samples. For trabecular analysis, a VOI spanning 15% of total femoral length for maternal bone and 7% for neonatal bone was placed at the metaphysis just proximal to the distal growth plate. Manual contours were used to isolate the trabecular compartment and further segmentation was performed using an automatic adaptive thresholding algorithm.

For vertebral analysis, a VOI was placed between the cranial and caudal endplates of maternal and neonatal L3 vertebrae. Vertebral cortical and trabecular bone were separated through manual contouring to denote the outer and inner boundaries of the cortex and segmented with automatic adaptive thresholding. The areas of interest were analyzed with CTAn software (version 1.15.4.0,

Bruker). Bone architecture parameters analyzed include: (trabecular number – TbN, trabecular thickness – TbTh, bone volume fraction – BV/TV, cortical thickness – CTh, and bone mineral density – BMD). Representative femoral and vertebral isosurfaces were obtained using commercially available software (MicroView Advanced Bone Analysis Application, GE Healthcare Pre-Clinical Imaging, London, ON, Canada).

Backscatter scanning electron microscopy

Previous studies have shown that osteocyte osteolysis plays an important role during lactation [123-126]. To assess physiologic osteocytic remodeling in our study, lacunar area was measured on maternal cortical bone of right femora. Following microCT imaging, maternal right femora were embedded without decalcification in methyl methacrylate and sectioned transversely below the lateral third trochanter using an Isomet low-speed diamond saw (Buehler Ltd., Lake Bluff, NY). Two hundred-micron sections were then polished and mounted on aluminum stubs using an alcoholic colloidal graphite solution. Images of osteocyte lacunae on the sectioned bone surface were acquired with a scanning electron microscope equipped with a backscattered electron detector (BSEM, Tescan MIRA3 FEG- SEM, Czech) at 30 kV accelerating voltage, 300 pA current, and 10 mm working distance. Acquired high contrast images were then converted into binary mask using ImageJ (NIH, Bethesda, MD) and areas were measured for all lacunae in the anterior, posterior, lateral and medial quadrant of the femoral cortex.

Biomechanical testing

To assess the mechanical effects during pregnancy and lactation and the influence of BPs during these events, maternal L5 vertebra and left femora were loaded to failure in compression and four-point bending, respectively, using an MTS 858 Mini-Bionix servo-hydraulic testing system (MTS

Systems Corp., Eden Prairie, MN, USA). All specimens were kept hydrated in lactated ringer's solution (LRS) prior to mechanical testing. The vertebral body was vertically aligned along its loading axis with an alignment pin (attached to lower platen and extending through the spinal column) and compressed to failure at a displacement rate of 0.05 mm/second. For four-point bending, the posterior surface of the femur was oriented in tension and the mid-diaphysis was loaded to failure at a displacement rate of 0.5 mm/second. Force and vertical displacements were continuously recorded throughout each test by a 50 lb load cell (Sensotec, Columbus, OH, USA) and an external linear variable differential transducer (LVDT; Lucas Schavitts, Hampton, VA, USA), respectively. A custom- developed LABVIEW program was used to calculate the mechanical properties for both tissues.

Tartrate-resistant acid phosphatase staining

Qualitatively, histochemical staining of the biochemical marker tartrate-resistant acidic phosphatase (TRAP) has been used to identify osteoclast activity. To assess TRAP activity in maternal and neonatal trabecular bone, right proximal tibiae were fixed (4% neutral buffered formalin), decalcified (10% EDTA for 21 days) and embedded in paraffin. Ribbons of serial sections (6μm) were cut with an automated Leica RM2255 (Leica, Wetzlar, Germany) rotary microtome and disposable low profile stainless steel blades (Accu-Edge, 4689). Collected sections were mounted on Superfrost/Plus glass slides (Fisher, Pittsburgh, PA., USA) and left to dry overnight on a 40°C slide warmer. Staining for TRAP was carried out with an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma- Aldrich, St. Louis, MO., USA) according to the manufacturer's instructions. Images were acquired using an upright microscope (Nikon Eclipse Ni-U) associated with a DS-Fi2 digital camera, NIS BR software (Nikon France, Champigny-sur-Marne, France) and a 20x dry objective. Histomorphometric analysis of maternal osteoclast activity was acquired

from two ROIs; the first was placed proximal to the growth plate at a span of 5 mm, while the second spanned 12.5 mm placed 5 mm distal to the growth plate. For neonates, due to high mineralized bone concentrated below the growth plate, a single 17.5 mm ROI was placed 12.5 mm distal the growth plate. In addition, neonatal osteoclast activity from mothers exposed to BPs at timepoints PC, E15 and D1 were analyzed to assess osteoclast activity on skeletal development when exposed to BPs through different pathways. When dams are treated with PAM PC, BP recycling resulting from bone resorption during periods of high calcium demand can expose offspring during both the embryonic or neonatal stage prior to weaning. Treating dams with PAM during gestation can lead to BPs to cross the placenta and expose the embryo to PAM (E15). Lastly, PAM treatment during lactation leads to potential diffusion of BPs into the breast milk, exposing neonates to PAM during a time they highly depend on their mothers for survival (D1). Analysis for both mothers and neonates was performed using Bioquant software (Bioquant Image Analysis Corporation, Nashville, TN., USA).

Statistics

Maternal bone architecture data are reported through box plots where treated (grey) groups are superimposed onto untreated (white) groups. Maternal variations in bone architecture, biomechanics, osteoclasts osteolysis, and osteoclast activity between untreated/treated groups and virgin controls were determined using a one-way ANOVA followed by Holm–Sidak test for multiple comparison. To determine differences between untreated and treated groups a two-way ANOVA was applied to analyze both maternal and neonatal parameters. Differences with p-values <0.05 were considered significant. All statistical analyses were carried out using Graph Pad Prism (version 7.04, San Diego, CA, USA)

Results

Maternal and neonatal body weight are not affected with pamidronate treatment

Maternal body weight increased during pregnancy (through E15), decreased immediately following birth (D1) and increased again through weaning (D15) (Figure 2.2A) in both PAM and PBS treated groups. By the end of lactation (D21), maternal body weight reached a common value regardless of PAM treatment status or time of treatment (Figure 2.2B). At 21 days of age, the body mass of genotyped female pups from PBS-treated dams did not differ from the body weight of genotyped female pups from PAM-treated dams. The same observations were seen in genotyped male pups. Furthermore, consistent with the reduced body size in OI, the body mass of HET pups was significantly lower than the body weight of WT pups in both females and males. This suggests that neonatal and maternal body mass was not affected by maternal PAM exposure during bone formation. The number of sudden deaths of pups in the litters was evenly distributed between PAM-treated (n = 9) and PBS-treated groups (n = 11), consistent with prior reports of spontaneous deaths in Brtl/+ pups ([64]). In addition, one PAM and two PBS treated dams were euthanized due to morbidity prior to the end of the experiment.

Pamidronate effects on pregnacy and lactation-induced bone changes are highly dependent on time of treatment

In Brtl/+ dams, pregnancy alone significantly increased vertebral Tb.N by 37% but significantly decreased Tb.Th by 10%, resulting in an overall increase in maternal bone volume fraction of 28% (Figure 2.3A; Treatment Timepoint PC; End Timepoint Birth) . The femur was less susceptible to these effects, as pregnancy had no effect on Tb.N but significantly decreased Tb.Th (7%), resulting in a 15% net bone loss (Figure 2.3B; Treatment Timepoint PC; End Timepoint Birth). Conversely, lactation induced a loss in Tb.N in both vertebra (7%) and femur (39%) as seen in Figure 2.3A and

Figure 2.3B (Treatment Timepoint PC-D15; End Timepoint Weaning). Together, these changes led to a 13% loss of vertebral bone volume fraction and an even greater femoral loss of 43% compared to virgin dams. When Brtl/+ dams were treated prior to conception with PAM, greater gains in vertebral Tb.N were obtained following pregnancy while no significant further gains were observed in the femur when compared to PBS- treated dams. Strikingly, when dams were carried out to the end of lactation (weaning of their pups at day 21), PAM induced a treatment-induced preservation of bone volume fraction through retained Tb.N that was directly related to timing of injection in both vertebra and femur. A modest preservation of bone mass was observed in the vertebra when PAM was administered prior to conception while significant gains of 58% were observed in the femur. PAM had no effect on Tb.Th regardless of time of treatment. In the cortical structure, pregnancy alone did not induce changes in vertebral or femoral C.Th, however, by weaned date, C.Th was reduced 16% and 10% in the vertebra and femur, respectively. Despite preservation of trabecular bone mass during the early stages of pregnancy and lactation (PC, E15 and D1) with PAM intervention, preservation of C.Th was only observed when PAM was administered prior to conception in both the vertebra (Figure 2.3C; Treatment Timepoint PC; End Timepoint Weaning) and the femur (Figure 2.3D; Treatment Timepoint PC; End Timepoint Weaning).

To qualitatively analyze the effects of PAM when administered at different stages of pregnancy and lactation, representative microCT images were extracted from virgin females and from dams at D21 post-weaning to visualize changes in maternal trabecular and cortical bone morphology. Lactation- induced bone loss was observed in both the vertebra and femur of PBS- treated dams (Figure 2.3E). When PAM was administered prior to conception, pregnancy and lactation-induced bone loss was completely prevented in Brtl/+ dams (Figure 2.3F). When PAM was administered

at E15 during pregnancy and D1 of lactation, a temporal preservation of bone mass was observed. No further preservation of trabecular structure was observed when dams were treated with PAM at D10 and D15 of lactation, suggesting trabecular bone loss had already occurred prior to this time point.

PAM-induced preservation of trabecular and cortical bone increases stiffness but not strength during pregnancy and lactation

Like the response seen in trabecular bone, a temporal effect was also observed in stiffness for both the vertebra and femur of PAM-treated dams. With pregnancy alone, a 57% increase in vertebral stiffness was observed in PBS-treated dams (Figure 2.4A), consistent with pregnancy-induced gains observed in Tb.N. No stiffness changes were observed in the femur following pregnancy alone, but a 10% femoral stiffness loss was observed by the end of lactation (Figure 2.4B). When PAM was administered prior to conception, vertebral stiffness in dams increased by 151% following pregnancy alone while a 50% increase was observed when assessed following lactation. Femoral stiffness assessed at birth and treated prior to conception showed an increase of 15% and an increase of 21% when assessed following lactation compared to virgin dams. Treatment with PAM induced a temporal effect on both vertebral and femoral stiffness when treated with PAM at different timepoints during pregnancy and lactation similar to the protective effect observed in their trabecular structure. Unlike stiffness, ultimate load in the vertebra did not show a temporal effect with PAM treatment. Instead, when dams were treated with PAM prior to conception and assessed at birth, a sustained effect was observed in vertebral max load, but an overall 26% was noted by weaned date regardless of PAM timepoint administration. Unlike the vertebra, a temporal effect with PAM treatment was observed in femoral ultimate load to failure when assessed at weaning. When dams were treated with PAM prior to conception a 6% increase in ultimate load

to failure was observed at birth, however, when assessed at weaning ultimate load decreased by 10% compared to virgin dams. This protective effect on femoral max load ceased to exist when PAM was administered at late stages of lactation.

BPs cross the placenta and diffuse into the breast milk

To assess if BPs cross the placenta, a fluorescent bisphosphonate imaging agent (Osteosense 680EX) was administered when Brtl/+ were in late stages of gestation (E19). As seen in Figure 2.5A fluorescent images of the fetal vertebral body showed localization of BPs during late stages of gestation, confirming that BPs can cross the placenta. Similarly, non-therapeutic BP was administered in Brtl/+ dams during early stages of lactation (D1) to assess if BPs diffuse into the breastmilk. In Figure 2.5B, fluorescent images of the maternal mammary glands verified the diffusion of BPs into the breastmilk.

Neonates at birth and weaning did not show skeletal adverse effects from maternal pamidronate exposure

Pregnancy and lactation represent a challenging period for the maternal skeleton as high demands of calcium are required for the healthy development of the neonatal skeleton. Since pregnancy and lactation-induced bone loss was prevented in PAM-treated dams, assessing neonatal skeletal development was imperative. At birth, following maternal PAM exposure during pregnancy alone, neonates (1 day of age) from dams treated with PAM prior to conception showed no effects from BP exposure during their early stages of skeletal formation as no differences in BMD were observed in either the vertebra or the femur (Figure 2.6A). Following maternal PAM exposure during pregnancy and lactation, at 21 days of age, sexed Brtl/+ and WT pups from PAM-treated dams continued to show no effect from BP exposure on trabecular and cortical structures (Figure

2.6B). To further assess that PAM exposure during pregnancy and lactation did not induce any adverse effects in the neonate's skeletal formation, Safranin O-stained sections reveal no morphological differences between pups from PBS and PAM treated dams (Figure 2.6C). Thus, despite preventing pregnancy and lactation-induced bone loss, no effects were observed in pups from PAM-treated dams compared to pups from PBS-treated dams.

Pamidronate increases maternal osteoclast number and surface but has no effect on neonates exposed to BPs during early stage of skeletal formation and lactation

To examine the presence of osteoclasts following maternal PAM exposure during pregnancy and lactation, mature osteoclasts positive for TRAP staining were identified in maternal tibia for all groups carried out through the end of lactation. TRAP+ mature osteoclasts (Figure 2.7A; N.Oc/BS) were present in both PAM and PBS-treated dams, albeit statistically more in PAM treated Brtl/+ dams. However, no differences were observed in osteoclast- surface coverage (Figure 2.7A; Oc.S/BS) between PAM and PBS-treated dams. To further assess the effect of the potential exposure to PAM during skeletal development, osteoclast activity in day 21 neonates from PBS and PAM treated dams was also analyzed following maternal intervention at prior to conception, E15 and D1. No differences in osteoclasts number (Figure 2.7B; N.Oc/BS) and surface coverage (Figure 2.7B; Oc.S/BS) between neonates from PAM and PBS-treated dams were observed suggesting that either our PAM dosage provides a low exposure risk during crucial periods of skeletal development or that PAM has not yet taken an effect on neonatal bone.

Pamidronate did not further increase osteocyte lacunar area during pregnancy

Backscattering imagining was used to analyze PAM effects on maternal osteocyte osteolysis during pregnancy and lactation at standard locations of the femoral cortical bone (Figure 2.8A).

Consistent with other studies, a significant increase in osteocyte osteolysis was observed following pregnancy in PBS- treated dams (Figure 2.8B). A similar increase was observed when dams were treated with PAM, suggesting that BP had no effect on lacunar size. By the end of lactation, when calcium demand is low, osteocyte osteolysis decreased and PAM continued to show no effect. Representative images of the differences in lacunar area under these conditions are shown in Figure 2.7C.

Discussion

The present study shows that pregnancy led to maternal gains in vertebral trabecular bone mass observed at parturition, while lactation induced maternal cortical and trabecular bone loss in both vertebra and femur observed at weaning in Brtl/+ mice harboring an OI-inducing Gly->Cys mutation in col1a1. BP intervention led to a temporal retention of maternal cortical and trabecular bone in both vertebra and femur. When PAM was administered prior to conception (PC), bone mass gains due to pregnancy were amplified and lactation-induced bone loss was prevented. This protective effect was more modest when given during pregnancy (E15) and ceased to exist in the late stages of lactation (D10 and D15). Furthermore, pregnancy induced osteocyte osteolysis which was unaffected with bisphosphonate treatment. Despite preventing lactation-induced maternal bone loss, no negative skeletal effects of BPs on offspring were observed. In view of the increasing use of BPs in premenopausal women to treat diseases causing high rates of bone remodeling like OI, these findings provide important clinical insight on a systematic window for when BPs are most effective in preserving maternal bone mass and how maternal exposure may affect embryonic and neonatal skeletal development.

Current knowledge on the effect of BPs during pregnancy remains limited and is based almost entirely on animal toxicity studies that show transplacental effects of BPs [79-82]. Administration of alendronate (0.1mg/kg) during gestation days 11-20 in rats resulted in BP accumulation in fetal bone which led to low birth weight and decreased bone growth [79]. Additionally, embryonic lethality, severe underdevelopment and fetal skeletal retardation was observed in a reproduction toxicity study involving rats and rabbits where PAM therapy was administered at a dosage 10x higher than what is used clinically [80]. In another similar rat study, BPs induced tremors, dystocia and death in dams during parturition as a result of pronounced maternal hypocalcemia [81]. These adverse effects are likely dose related, however fewer studies exist that examine sub-toxic doses. No adverse maternal or offspring effects were observed in a rat and rabbit study using low dosages of icadronate, however when dosage was increased, dam death, retarded fetal ossification and abnormal tooth growth were noted [82]. Human data on BP effects during pregnancy is also limited. Scattered clinical cases reported uneventful pregnancies under BP treatment [68, 85, 106], but a few case studies did show lower gestational age at birth, small birth weight and increased rate of spontaneous abortions [127]. However, due to the rarity of these outcomes it was speculated that the effects could have been a result of maternal underlying conditions rather than BP itself [127].

To date, two animal toxicity studies have explored the effects of BPs during lactation. Both bovine and rat studies showed that BPs induce hypocalcaemia in response to the inhibition of lactation-induced bone loss [89, 113]. In two pharmacological studies, BP treatment attenuated but did not prevent lactation-induced bone loss, however, no neonatal effects were reported [128, 129]. Several studies have shown evidence of post-lactation recovery of maternal bone loss [130-132] which may occur independent of preceding resorption events. When zoledronate was administered

at the beginning of lactation, bone formation persisted after forced weaning despite prevention of lactation-induced bone loss [133]. With regards to human data, there is a dearth of case studies reporting the effects of BPs when administered prior to, or during lactation. Of the case studies that are available, both mother and infant did not demonstrate serious adverse effects [117, 119, 127, 134], however one report cited transient asymptomatic hypocalcaemia of the newborn [116].

The present study differs from prior animal studies by using sub-clinical doses compared to the high doses previously used to evaluate BP safety during pregnancy and lactation. Due to the differing bone metabolic effects during pregnancy and lactation, our approach was to identify a window for when BPs are most effective at inhibiting bone loss without disrupting calcium homeostasis for fetal and neonatal growth in an OI mouse model. In the present study, pregnancy triggered a significant increase in trabecular bone volume fraction of Brtl/+ dams, similar to results observed in other animal studies [135, 136]. However, this increase in bone mass was only observed in the vertebral body as no significant differences were noted in the distal femur. During pregnancy, studies have shown that there is an accumulation of calcium in the maternal skeleton to serve as a calcium reservoir during lactation [137]. Other animal studies have also reported that following conception, there is an immediate accumulation of skeletal mass and its utilization begins around late pregnancy when the skeleton begins to form in the developing fetus [138, 139]. This process is then followed by a decline in maternal bone mass during lactation, as the neonatal skeleton begins to mineralize [140, 141]. Similar observations were observed in the present study, as trabecular bone mass significantly decreased in both the vertebra and distal femur during lactation. Although pregnancy did not induce any response on cortical bone, significant thinning of the cortical bone was observed following lactation, consistent with results reported in other studies [137, 140, 142].

Several studies have established that treating OI patients with BPs can decrease bone turnover and increase bone mineral density [20-26]. We have also shown in previous studies that BPs increase trabecular bone mass during growth through retention of trabecular number [53, 120, 121]. In this study, PAM intervention prior to conception (PC) prevented lactation-induced bone loss solely through retention in Tb.N as a net decrease in Tb.Th was observed with or without treatment. Retention of Tb.N was more modest when PAM was administered during pregnancy and early stages of lactation (E15 and D1) and ceased to exist in the late stages of lactation (D10 and D15). A less robust temporal effect was observed in cortical bone. These findings helped confirm that bone retention was clearly dependent on time of PAM administration.

Increase in Tb.N following pregnancy drove gains in vertebral stiffness that were amplified with PAM intervention prior to conception. When the maternal skeleton was assessed following lactation, inhibition of lactation-induced bone loss with PAM when administered prior to conception (PC) led to significant preservation of vertebral and femoral stiffness. Similar observations were seen with PAM intervention during pregnancy (E15) and early stages of lactation (D1), while no differences were noted between treated and untreated dams at later stages of lactation (D10 and D15). Although no differences were observed in vertebral maximum load between treated and untreated dams when assessed at the end of lactation, a temporal effect was observed on femoral maximum load.

Since we confirmed through FRFP that BPs were able to not only cross the placenta, but also diffuse into the breast milk and that PAM intervention prevented lactation-induced bone loss in maternal bone, it was vital to assess if fetal and neonatal bone development was affected as well. Surprisingly, vertebral and femoral BMD of newborn WT and Brtl/+ mice (1 day of age) was unchanged when BP was administered prior to conception or during the embryonic stage. We

propose several possible means to alter fetal and neonatal bone metabolism through fetal and neonatal BP exposure, as well as altered maternal bone metabolism. Embryonic bone ossification has been reported to start near gestation day 14 [143]. Therefore, administration of PAM during early stages of fetal bone ossification may lead to BP exposure through placental crossing and localization on mineralized fetal bone during the embryonic stage. During lactation, it has also been well established that the demand for calcium is greatest at the beginning and decreases as offspring are introduced to outside food sources [130, 144]. As a result, when the maternal skeleton was treated with PAM at either D1, D10 or D15 of lactation, risk of exposure through diffusion into the breast milk was likely greatest at the beginning of lactation. Because of the long half-life of BPs, treating dams prior to conception may expose fetal and neonatal bone to PAM through bisphosphonate recycling secondary to maternal bone resorption. Alternatively, PAM can indirectly interfere with fetal and neonatal development secondary to inhibition of maternal bone resorption. Direct disruption of maternal calcium homeostasis through anti-resorptive effects may prevent offspring from receiving the necessary calcium supply for healthy bone development. Despite these potential exposures to PAM, Brtl/+ and WT offspring (21 days of age) from treated and untreated mothers showed no differences in trabecular bone mass or cortical thickness. These findings suggest that the single PAM exposure had no effect on skeletal development whether potentially transferred through the placenta, breast milk or BP recycling resulting from maternal bone resorption, or through altered maternal calcium homeostasis.

It has been well established that for successful reproduction, it is vital that the mother gains an appropriate amount of weight during her pregnancy to provide adequate nutrients to allow the fetus to grow. In mice, pregnancy has been linked to hyperphagia resulting in linear weight gain until parturition [145]. However postpartum, studies have shown that pups begin eating solid food

midway through lactation which reflects a decrease in maternal weight and milk production [146]. We observed a linear increase in maternal weight during pregnancy and the first half of lactation. When maternal body mass was assessed at weaning, no differences were observed between PAM and PBS- treated dams, suggesting that BP exposure did not influence maternal food intake. Additionally, we also observed no adverse effects of PAM on neonatal weight at weaned date. These findings are in agreement with a study that evaluated the dependency of lactation-induced bone loss and post-lactation bone gains using zoledronate treatment where no adverse effects were observed on body weight of dams or ash weight of pups [133].

We observed no difference in osteoclast surface or number in maternal tibiae between virgin controls and PBS-treated dams following lactation, despite the trabecular bone loss observed during this time. It is possible that we missed the window of high osteoclast activity since assessment was performed at time of weaning when bone turnover rate has slowed down (as seen from our D10 and D15 treated dams), however, no osteoclast assessment was performed following parturition. To support these findings, there is accumulating evidence that at the end of lactation there is a rapid reversal in maternal bone metabolism where bone formation greatly increases to begin to recover the maternal skeleton and rebuild its mineral storage in preparation for the next reproductive period [147]. In fact, female mice are in fertile estrus about 10 and 24 hours after giving birth, suggesting that this window of recovery is particularly short in mice. Furthermore, osteoclasts become apoptotic immediately with the cease of lactation [148]. Similarly, we did not observe an effect of PAM on osteoclast surface coverage, however greater osteoclast numbers were observed with treatment. In rats treated with a high dose of zoledronate, the number of osteoclasts increased significantly, and a number of other studies have shown that the number of osteoclasts could stay the same or even increase during BP treatment [149-151]. Alternatively,

another study showed that treatment with zoledronic acid reduced osteoclast surface when administered for both short and long durations [152].

We assessed osteoclast activity in the neonates at wean date since it would allow us to evaluate the potential risk of PAM exposure on the role of octeoclasts during the ossification and mineralization stages of bone development. We explored timepoints PC, E15 and D1 since not only was maternal bone loss inhibited with PAM at these time points, but these time points dictate three possible routes of fetal and neonatal BP exposure. Considering that no differences on osteoclast surface and number were observed, we can conclude that in this model, BP exposure through recycling (PC), placental crossing (E15) and diffusion into the breast milk (D1) have no effect on embryonic and neonatal development. These limited effects might be due to two possibilities; embryonic and neonatal bone formation rate may exceed bone resorption, so osteoclasts don't play a significant role during these early stages of bone development; our single PAM dosage was not enough to induce a significant biological effect.

Although osteoclasts have been assumed to be primarily responsible for the removal of bone during calcium homeostasis, osteocytes have also been found to play a role during periods of increased calcium demand. While some studies have shown osteocytes play a role in calcium homeostasis during pregnancy and lactation [125, 153] others have not [154]. In our study, following pregnancy, Brtl/+ dams showed an increase in lacunar area which returned to baseline by end of lactation. While these changes were modest (16%) it is possible that lacunar area was higher during the earlier stages of lactation. Prior studies have shown osteocytic perilacunar and canalicular remodeling on the twelfth [124] and fourteenth day of lactation [155] while our observations were limited to parturition and weaning only. Most importantly, although maternal bone loss was prevented with PAM intervention, PAM showed no effect on osteocyte lacunar

remodeling during this crucial period of calcium demand. Conversely, others have shown inhibition of osteocyte osteolysis during lactation through calcitonin [156] and parathyroid hormone [157], which may reflect a direct action on the osteocytes through related signaling pathways.

This study has several limitations. To assess history of BP therapy, a single PAM dosage was administered prior to conception, which does not reflect continuous BP accumulation in the maternal skeleton due to extensive treatment prior to conception in humans. Exploring a wider range of dosages will be important to fully assess the risk/benefit ratio of bone interventions during pregnancy and lactation. It is now well established that BPs have a long half-life and remain embedded in the bone matrix until liberated during osteoclastic resorption. Thus, fetal and neonatal BP effects might not be observed until later stages of rapid growth or adulthood when BPs are recycled through bone remodeling. Therefore, although no effects were observed on weaned pups, BP exposure risk should not be disregarded, and post-lactation assessment of neonatal growth should be considered. Despite inhibiting lactation-induced maternal bone mass, no effects were observed in fetal and neonatal bone development. However, we did not analyze calcium levels in mothers or their offspring to verify that PAM did not indirectly interfere with fetal and neonatal development secondary to inhibition of maternal bone resorption. Since it has been well established that pregnancy up-regulates intestinal calcium absorption [158], potential absorption changes in the presence of an anti-resorptive need to be addressed. Furthermore, treatment timing will vary depending on fracture risk diagnosis during pregnancy and lactation to attain the best clinical outcome.

Aside from studying the effects of bone therapeutics in animal models for their translation into human clinical trials, studying any therapeutic effect during pregnancy and lactation raises several

challenges particularly since mice have a shorter gestation period (18 to 21 days) than humans (280 days). In this study, PAM was administered in mice on gestation day 15 which has been shown to be equivalent to week 8 of pregnancy in humans [159]. Although this timepoint represents the commencement of ossification in both timelines [159, 160], the rapid skeletal development in mice significantly reduces their risk of possible transplacental exposures. As a result, PAM intervention in humans might lead to greater exposure risks in utero because 80% of their gestation period remains as oppose to 29% in mice. Once born, there is a wide variation in the developmental phases between mice and humans and correlating their relative ages can be determined through several factors [161]. For example, mice are weaned at day 21 post-birth while on average the weaning age for humans is about 6 months (180 days) [162]. Thus, in this study, neonatal mice were exposed to PAM on days 1, 10 and 15 of lactation which are equivalent to 9, 86 and 129 days of age in humans, respectively. However, this correlation through weaned date may not accurately represent the metabolic bone state between both timelines, suggesting that further studies are necessary to assess these limitations.

During pregnancy and lactation, significant demands are placed on the maternal skeleton to provide the necessary calcium supply required for fetal and neonatal bone formation. The changes in calcium homeostasis during these reproductive periods can influence a time-dependent BP effect on bone metabolism that is not captured in toxicology studies. The results in this study capture the temporal effects on bone metabolism when PAM was administered at different stages of pregnancy and lactation. Specifically, when the maternal skeleton was treated with PAM prior to conception, lactation-induced bone loss was prevented. Despite inhibiting lactation-induced bone loss with PAM treatment, no adverse effects were observed on neonatal bone development. Additionally, our results of osteocyte remodeling provided further insight to their contribution to

the regulation of calcium homeostasis during periods of high calcium demand from the maternal skeleton. We believe that calcium release from trabecular thinning and osteocyte osteolysis during pregnancy and potentially intestinal absorption (though not tested in this study) may have contributed to the healthy neonatal bone development observed here. These results offer a treatment window during pregnancy and lactation for when BPs are most effective and represent a further significant insight of the maternal changes in bone metabolism due to calcium homeostasis during this reproductive period.

Figures

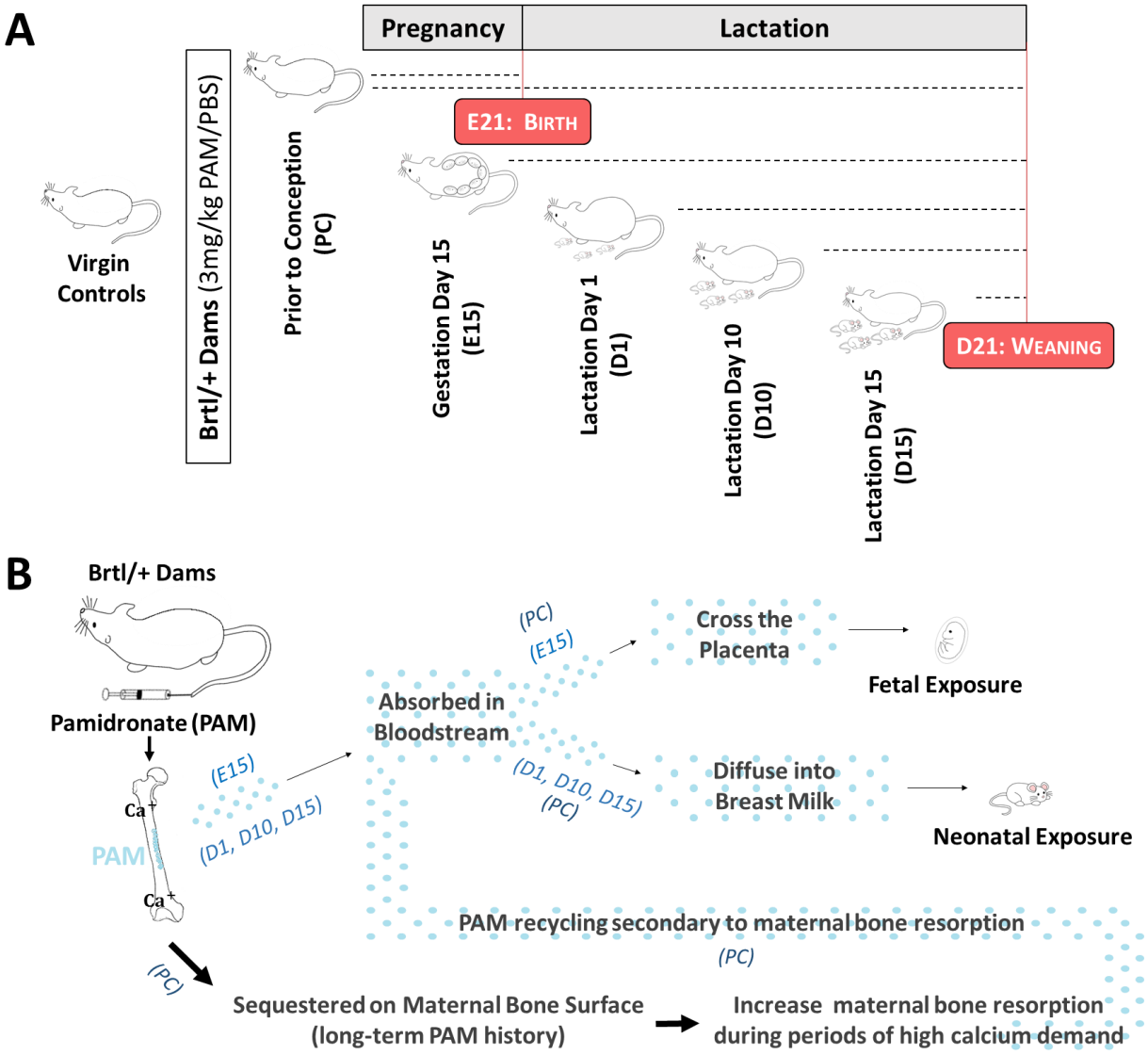

Figure 2.1: *Assessing PAM effects at different stages of pregnancy and lactation in the maternal skeleton and its effects on offspring bone development from PAM exposure.* A) Single injection of PAM treatment or PBS was administered during pregnancy and lactation in Brtl/+ dams. Virgin controls were used to establish a skeletal baseline uninfluenced by these reproductive periods. B) Proposed pathways of PAM exposure on fetal and skeleton bone development when the maternal skeleton is treated with BPs during the different stages of pregnancy and lactation. Not shown, potential indirect effect on calcium homeostasis from osteoclastic inhibition of maternal bone resorption during fetal and neonatal bone development.

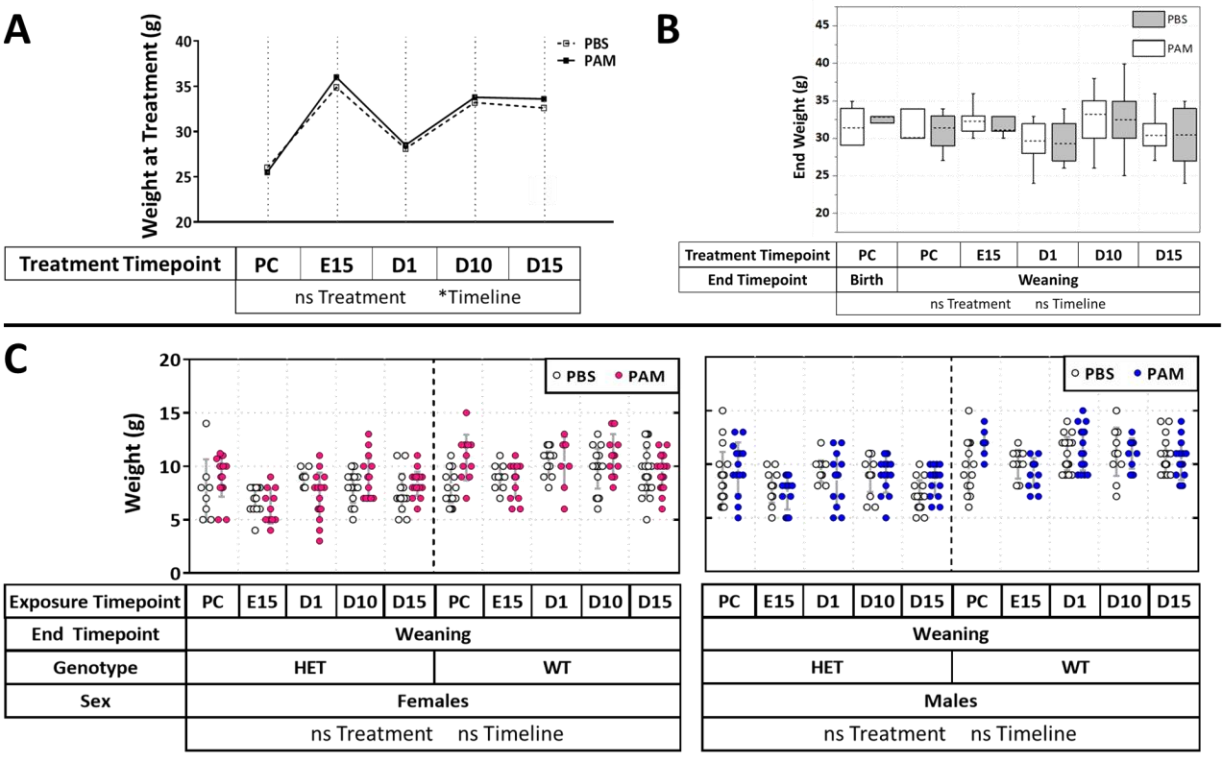

Figure 2.2: *Maternal and neonatal body weight are not affected with pamidronate treatment. A) Maternal body weight increased due to pregnancy and lactation and no body weight differences between PBS and PAM treated groups were observed. B) Greater gains in maternal body weight were not induced with PAM treatment. C) No differences in body weight of pups from PAM-treated dams and PBS-treated dams were observed. Results of Two-Way ANOVA factors: * p<0.05.*

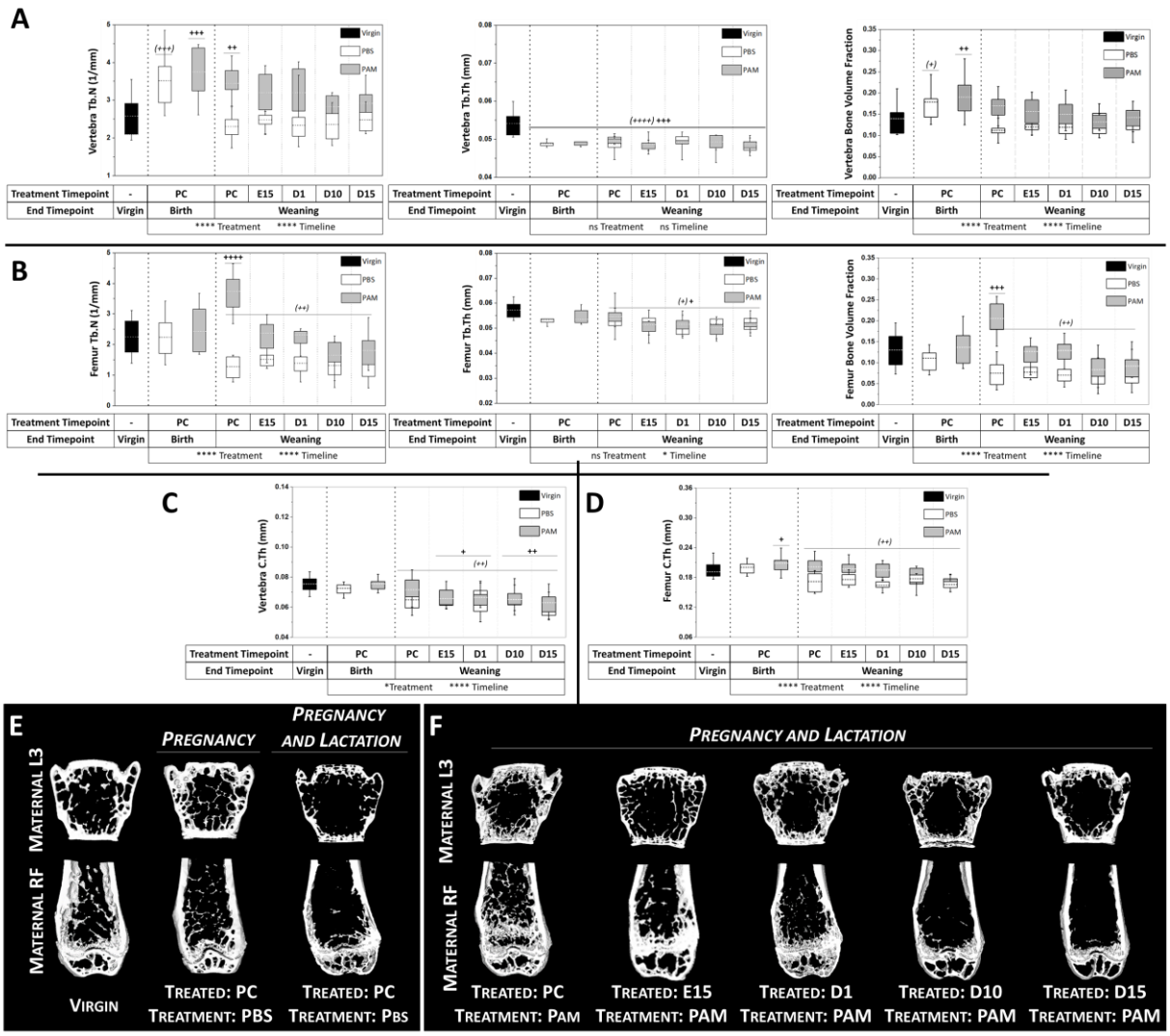

Figure 2.3: *Pamidronate effects on pregnancy and lactation-induced bone changes are highly dependent on time of treatment.* MicroCT analysis of the maternal Brtl/+ skeleton showed a temporal response to PAM intervention during pregnancy and lactation in both vertebra (A) and femur (B). A less robust temporal effect was observed in maternal cortical bone of both vertebra (C) and femur (D). Representative microCT isosurfaces of the distal femur metaphysis and the vertebral body reflect maternal bone structure in response to pregnancy and lactation in both untreated (E) and treated dams (F). Results of One-Way ANOVA factors: **PAM** and (PBS) compared to virgin controls: ++++ $p<0.00005$, +++ $p<0.0005$, ++ $p<0.005$, and + $p<0.05$. Results of Two-Way ANOVA factors: **** $p<0.00005$, *** $p<0.0005$, ** $p<0.005$, and * $p<0.05$.

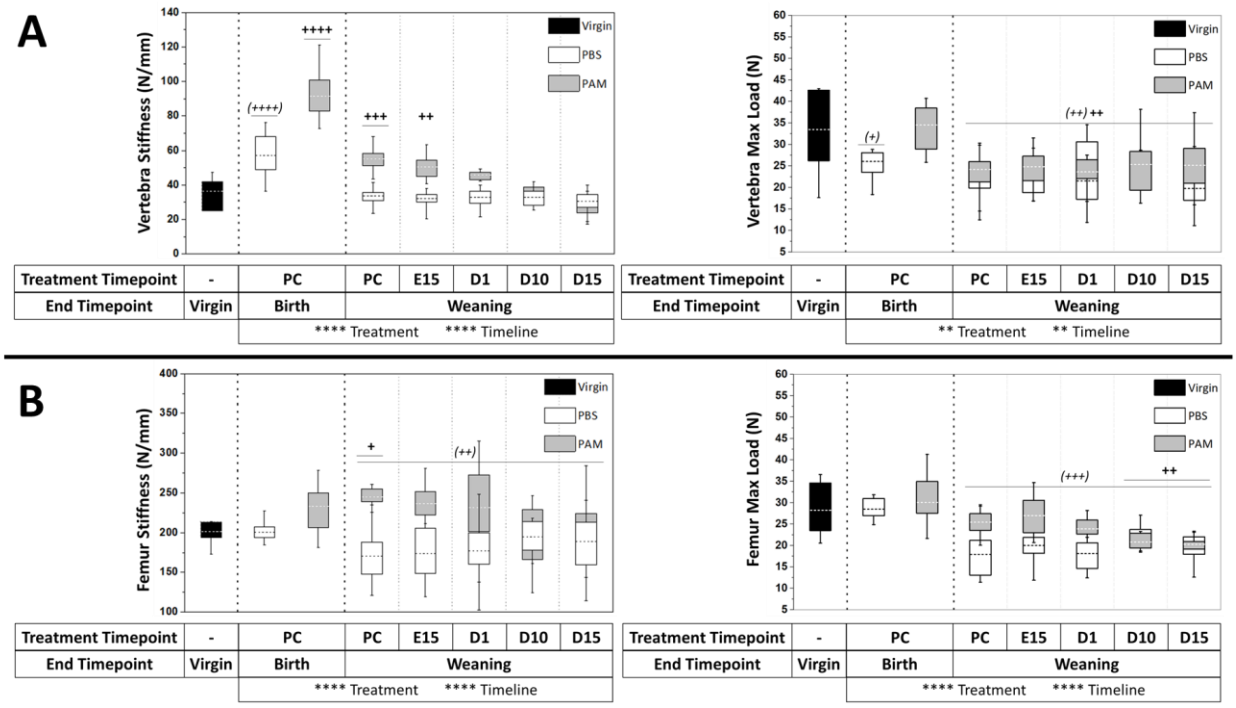

Figure 2.4: *PAM-induced preservation of trabecular and cortical bone increases stiffness but not strength during pregnancy and lactation.* A) With PAM treatment PC, significant gains in vertebral stiffness were observed following pregnancy and lactation but no preservation in maximum load to fracture was observed. Similarly, in the femur, PAM showed a sustained effect on stiffness and also a loss in maximum load to fracture (B). Results of One-Wa4.y ANOVA factors: **PAM** and (PBS) compared to virgin controls: ++++ $p<0.00005$, +++ $p<0.0005$, ++ $p<0.005$, and + $p<0.05$. Results of Two-Way ANOVA factors: **** $p<0.00005$, *** $p<0.0005$, ** $p<0.005$, and * $p<0.05$.

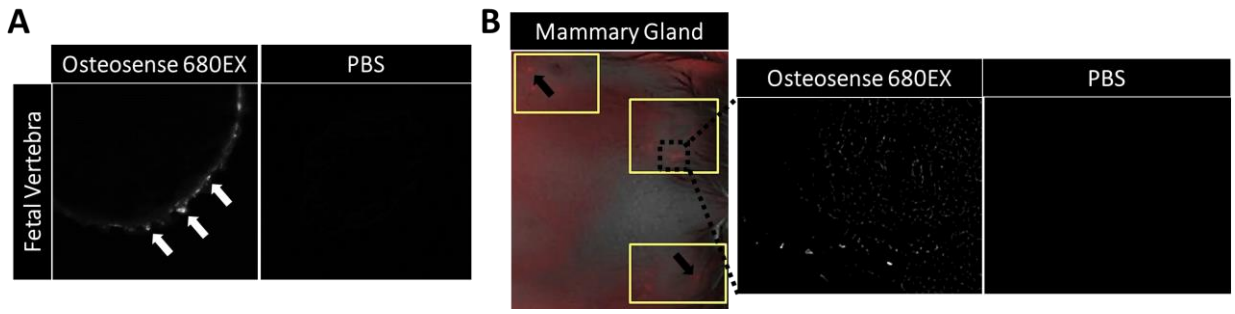

Figure 2.5: *BPs cross the placenta and diffuse into the breast milk.* A) Fluorescent non-therapeutic BPs localized in fetal vertebral body during late stages of gestation and in the B) mammary gland during lactation. Control tissues from animals receiving PBS and imaged under identical acquisition settings show negative fluorescence.

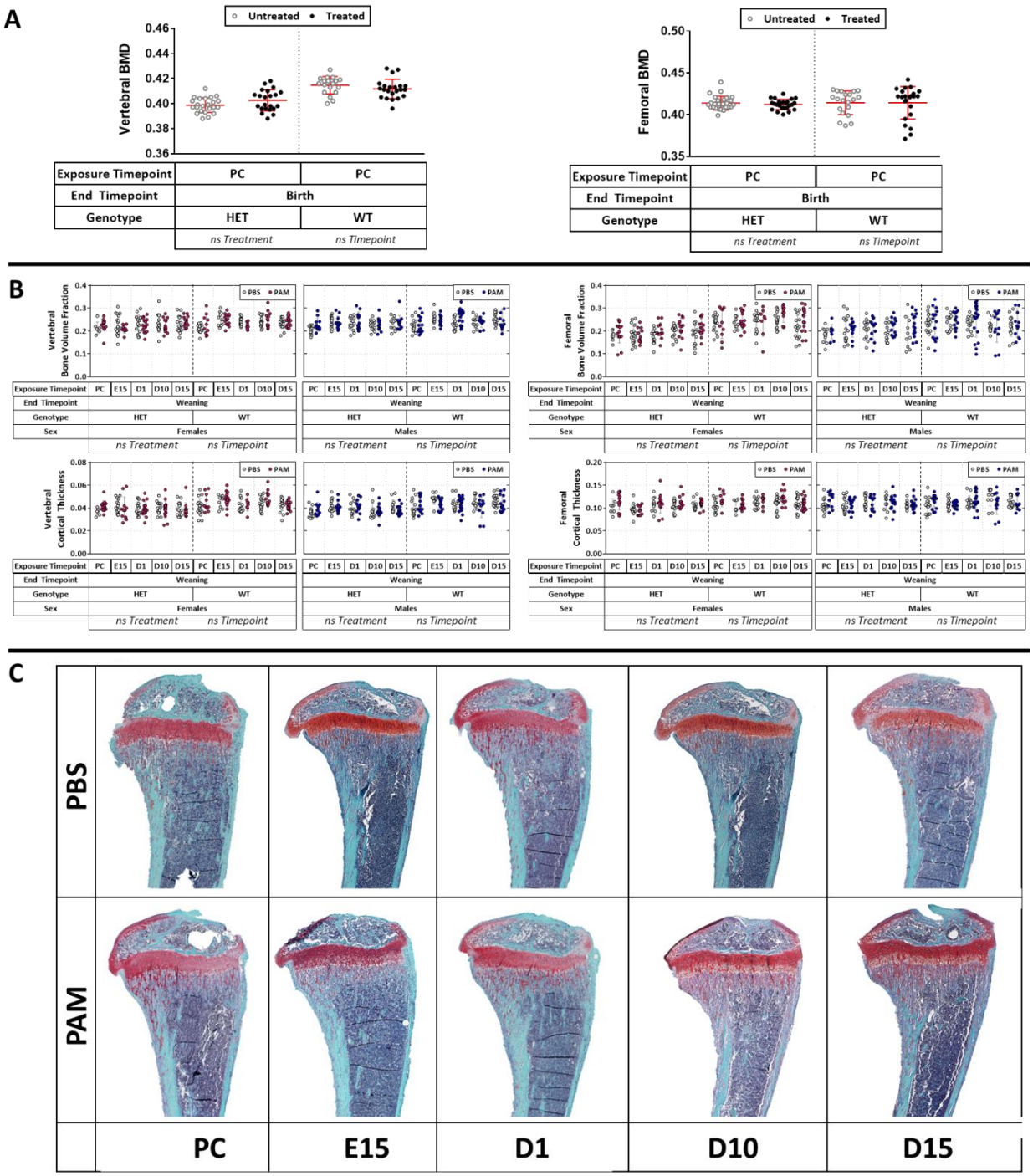

Figure 2.6: *Neonates at birth and weaning did not show skeletal adverse effects from maternal pamidronate exposure.* A) No changes in vertebral and femoral bone mineral density were observed at birth in sexed Brtl/+ and WT offspring following PAM exposure during the embryonic stage. B) At 21 days of age, sexed Brtl/+ and WT neonates from PBS and PAM treated dams showed no change in bone mass or cortical thickness when exposed to PAM at different stages of their skeletal development. C) Representative histomorphometric images of neonatal femurs show

no adverse effect in bone formation from maternal PAM exposure. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.

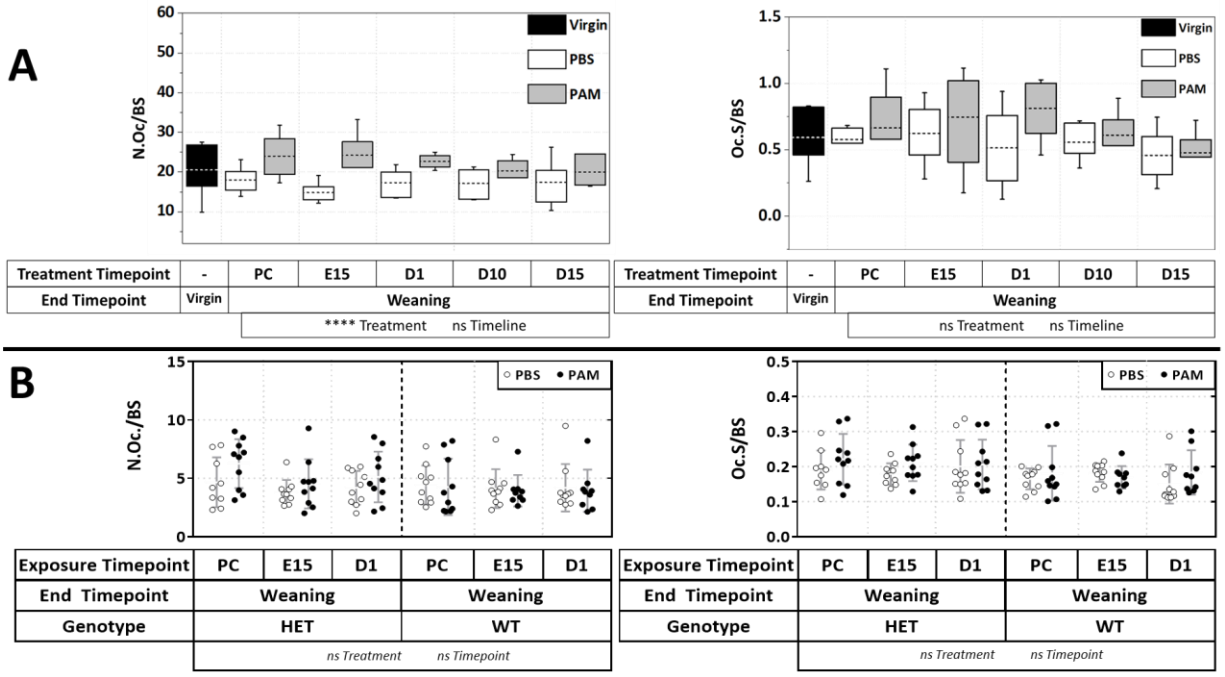

Figure 2.7: *Pamidronate increases maternal osteoclast number and surface but has no effect on neonates exposed to BPs during early stage of skeletal formation and lactation.* A) Histomorphometric quantification of TRAP-stained tibial bone sections showed that PAM treatment increased maternal osteoclast number and surface coverage compared to PBS-treated dams. No temporal effect was observed with PAM treatment. B) Osteoclast activity in pups from PAM- treated dams was not significantly altered with BP exposure at PC, E15 and D1 compared to pups from PBS- treated dams. Results of One-Way ANOVA factors: **PAM** and (PBS) compared to virgin controls: ++++ p<0.00005, +++ p<0.0005, ++ p<0.005, and + p<0.05. Results of Two-Way ANOVA factors: **** p<0.00005, *** p<0.0005, ** p<0.005, and * p<0.05.

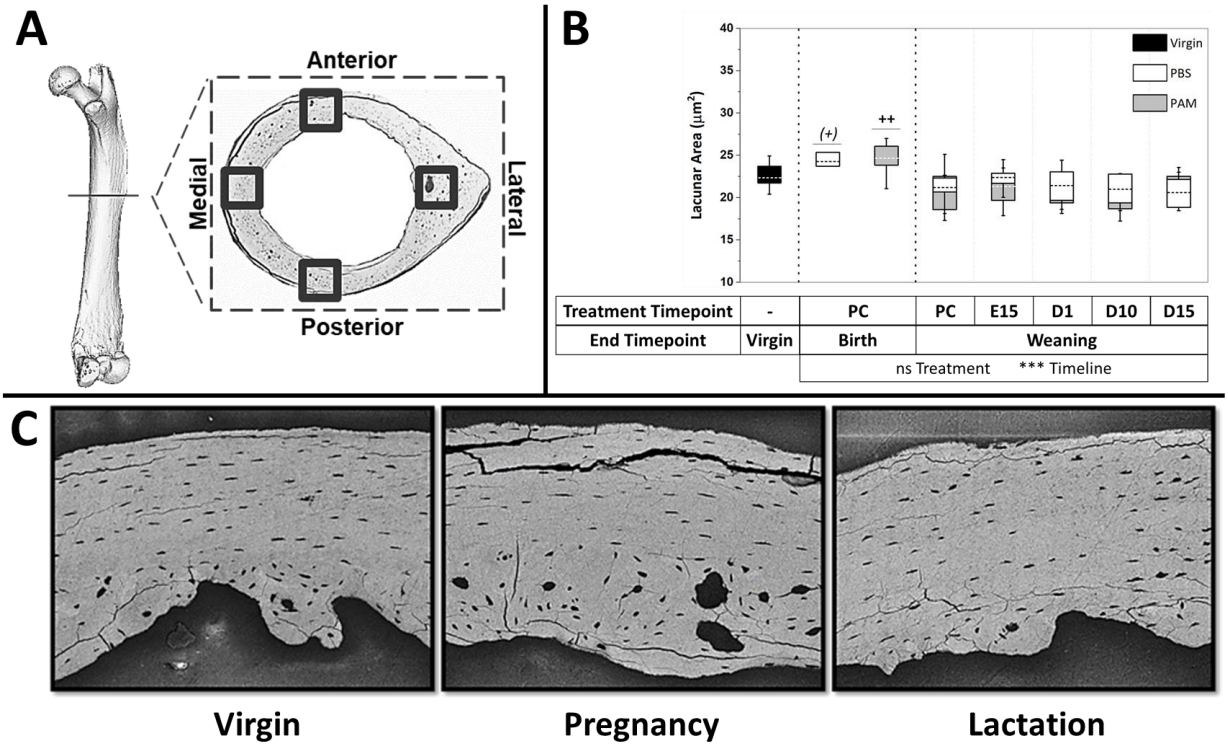

Figure 2.8: *Pamidronate did not further increase osteocyte lacunar area during pregnancy.* A) Panels show the location of measurements and representative images of lacunae from PBS and PAM treated dams. B) Pregnancy induced osteocytic lacunar enlargement in the femur as measured by backscatter electron microscopy. By the end of lactation, osteocyte lacunar area remodeled back to baseline. Results of One-Way ANOVA factors: **PAM** and (PBS) compared to virgin controls: ++++ $p<0.00005$, +++ $p<0.0005$, ++ $p<0.005$, and + $p<0.05$. Results of Two-Way ANOVA factors: **** $p<0.00005$, *** $p<0.0005$, ** $p<0.005$, and * $p<0.05$.

Chapter 3 Low Dose of Bisphosphonate Enhances Sclerostin Antibody-Induced Trabecular Bone Mass Gains in Brtl/+ Osteogenesis Imperfecta Mouse Model

Introduction

Osteogenesis imperfecta (OI) is a genetic bone disorder caused by collagen-related mutation resulting in type dependent skeletal phenotypes ranging from subclinical to lethal severity. [4] Though these phenotypes are type-dependent, OI is most commonly associated with low bone mass, altered bone quality, and imbalanced bone remodeling leading to skeletal fractures and deformities such as scoliosis, short stature, and bowing of the long bones [163]. In the presence of OI, upregulation of osteoclast activity causes a cellular imbalance that favors resorption, resulting in thinner bones with fewer trabeculae, both of which greatly increase fracture risk during childhood. [164]

Despite no current cure for the disease, treatments for pediatric OI have focused on improving bone density to promote functional strength and consequently reduce bone fragility. Currently, antiresorptive agents from the class of bisphosphonates (BPs) are the standard of care for pediatric OI. Through their high affinity for calcium ions, these potent inhibitors of bone resorption strongly bind to hydroxyapatite bone surfaces where they can later be internalized by osteoclasts to interrupt the resorption process. [165] Several controlled clinical trials have established the beneficial effects of treating pediatric OI with BP, including decreased bone turnover and increased bone mineral density, particularly at sites of trabecular bone. [22, 32, 166-170] During endochondral growth, primary trabeculae are remodeled and converted to secondary spongiosa, and extended

BP treatment interrupts this process, thus retaining calcified cartilage and increasing metaphyseal mass. In support of these studies, we and others have shown that with BP treatment, significant improvements in trabecular number, but not trabecular thickness are realized. [28, 96, 171-174] However, there remain concerns about the long-term treatment and retention of BPs in a growing skeleton, [175] and there remains a clinical need for additional therapeutic strategies that can minimize BP dose while maximizing therapeutic benefit.

Recently, sclerostin antibody (SclAb) has gained interest as an anabolic approach for the treatment of OI. [47, 48, 50, 53, 176, 177] Sclerostin, expressed in mature osteocytes, inhibits bone formation by exerting antagonistic effects on Wnt signaling and downstream osteoblast activity. [178] We have previously shown a significant anabolic response to SclAb in an OI mouse model. [47, 48, 50, 53, 176] These studies showed that treatment with a neutralizing antibody to sclerostin stimulated bone formation in cortical and trabecular bone, resulting in significant improvements in biomechanical properties. Unlike BPs, SclAb led to significant improvements in trabecular thickness. In the present study we sought to determine whether BPs could be used to augment SclAb efficacy by inducing retention of primary trabeculae, which could then serve as a substrate for the anabolic actions of SclAb to increase trabecular bone mass. We hypothesized that when combined, these two interventions would independently target different pathways of the bone remodeling cycle, leading to increases in trabecular bone mass greater than when either therapy was given alone. To accomplish this, in the present study, we test the immediate and long-term effects of treating rapidly growing Brtl/+ mice, harboring an OI-causing defect, with SclAb and BP together during growth. Our knock-in mouse model reproduces features of moderately severe Type IV OI. Treatment of Brtl/+ with alendronate alone increased trabecular bone mass through retention of calcified cartilage, with modest cortical gains that did not translate directly to

biomechanical improvements. [174] Conversely, treatment of Brtl/+ with SclAb alone elicited significant gains in both trabecular and cortical bone mass. [47] In the present study, through combination therapy, SclAb and BP induce gains in both trabecular thickness and number, leading to synergistic gains in trabecular bone stiffness, suggesting a distinct advantage to combination therapy in a growing mouse model of OI.

Materials and Methods

Animals

Wild-type (WT) and Brtl/+ mice with a mixed background of SV129/CD-1/C57BL/6S were derived from heterozygous Brtl/+ and WT parental strains. [64] To assess the short-term effects of pamidronate (PAM) and SclAb combined, at 21 days of age, male WT and Brtl/+ mice received a single intraperitoneal injection of PAM at either 0.3 mg/kg or 0.625 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) or saline control. These doses of PAM represent 10% and 20% of doses shown to induce trabecular retention for 20 days in mice of similar age. [96] After a 3-day latency period, mice were randomly assigned to SclAb treatment (Scl-Ab VI, 25 mg/kg; Amgen, Thousand Oaks, CA, USA) or saline groups and injected subcutaneously twice a week for 2 weeks (through day 34). SclAb was chosen at a dose that consistently induced an anabolic effect in our prior studies. [47, 48, 50, 53, 176] Calcein and alizarin were administered 1 day before PAM and at day 35, respectively, by intraperitoneal injection (30 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) to visualize the growth pattern during treatment. Short-term treatment mice (n = 7/group) were euthanized at day 37. Similarly, to evaluate the long-term effects of PAM and SclAb combined, male WT and Brtl/+ mice received two cycles of combination therapy, repeating the treatment

described above on day 38. Calcein, alizarin, and calcein were administered 1 day before cycle 1, 1 day before cycle 2, and at day 53, respectively, by intraperitoneal injection (30 mg/kg). Mice (n = 10/group) were euthanized at day 55. A summary of the experimental design for short-term and long-term assessment of combination therapy is shown in Figure 3.1A, and Figure 3.1B respectively. A schematic of our proposed mechanism of action for combination therapy is shown in Figure 3.1F. All protocols and procedures were approved by the University of Michigan's Committee on Use and Care of Animals.

Micro–computed tomography

To determine the short and long-term effects of PAM and SclAb on trabecular and cortical bone morphology, L5 vertebrae and left femurs were scanned in water via micro–computed tomography (mCT) (eXplore Locus SP; GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Scans were performed with the source set at 80 kVp and 80 µA using the Parker method of rotation (180 degrees plus a 20-degree fan angle), 0.5-degree increment angle, four frames averaged, and an acrylic beam flattener with a 0.02 Al filter were used for filtration of beam hardening artifacts. Images were reconstructed at a voxel size of 18 µm, and a hydroxyapatite-mimicking phantom was used to calibrate grayscale values for densitometry. [179]

Treatment of the growing skeleton with antiresorptive agents induces formation of a BP-laden band reflecting retention of primary spongiosa in the femur. [180, 181] To study the short-term combined influence of PAM and SclAb on the femur, a 0.7-mm ROI was placed at this BP-induced retention region of the distal femur. A second ROI was located distal to the band (closer to the growth plate), representing bone formed subsequent to BP injection, during SclAb treatment. A third ROI was located proximal to the band representing a site of trabecular bone formed prior to

BP injection, but still under the influence of SclAb. A summary of the position of these ROIs are shown in Figure 3.1C. Segmentation and binarization of trabecular bone at these sites was performed using a local threshold adjusted at each site for optimal representation of trabecular microarchitecture. Trabecular bone at and distal to BP band was segmented at 1500 Hounsfield units (HU), while trabecular bone found at the region proximal to BP band was segmented at 1300 HU.

To understand the resulting effects from concurrent cycles of combination therapy in the femur, a 5-mm ROI was placed at the metaphysis just proximal to the growth plate and extending through to the location of the proximal-most band of retained trabecular bone. To isolate the effects of each administered cyclic treatment, this full cycle ROI was broken down into representative regions of cycle 1 (2.7 mm) and cycle 2 (1.8 mm). Cycle 1 spanned from above the first BP band to right above the second induced BP band. Cycle 2 spanned above the second sclerotic BP band to right above the growth plate. A schematic of the placement of these three ROIs is shown in Figure 3.1D. Due to the maturity of the bone and size of ROIs, a local auto-threshold was used to quantify trabecular bone at these three regions. [182]

Although a retention-related band was observed in the femur following BP treatment, a "bone in bone" appearance was induced in the vertebral body, consistent with vertebral growth patterns and clinical observations. [183] As a result, an ROI was placed between the cranial and caudal endplates of the L5 vertebrae to analyze both short-term and long-term effects of combination treatment (Figure 3.1E). Vertebral cortical and trabecular bone were separated through manual contouring to denote the outer and inner boundaries of the cortex and segmented by a local auto-threshold.

To measure both short-term and long-term effects of treatment on femoral cortical bone, an ROI spanning 15% of total femoral length was centered midway between the lateral third trochanter and the distal femoral growth plate in all samples. A local auto- threshold was used to quantify cortical bone. Bone architecture parameters (trabecular number [Tb.N], trabecular thickness [Tb.Th], bone volume fraction [BV/TV], and cortical thickness [C.Th]) were analyzed using commercially available software (MicroView Advanced Bone Analysis Application; GE Healthcare Pre-Clinical Imaging, London, ON, Canada).

Bone histomorphometry

To qualitatively analyze bone formation response from short-term and long-term combination therapy, femurs and L2 vertebrae were embedded without decalcification in methyl methacrylate. Both tissues were sectioned longitudinally with a Reichert-Jung Polycut microtome (Reichert-Jung, Heidelburg, Germany) at a thickness of 8 µm and mounted on gelatin-coated glass slides. An upright microscope (Nikon Eclipse Ni-U, Tokyo, Japan) associated with a DS-Fi2 digital camera and NIS BR software (Nikon, Champigny-sur-Marne, France) was used to acquire calcein and alizarin fluorescent images with a 10x dry objective.

Biomechanical testing

To assess the mechanical effects of short-term and long-term combination therapy, L5 vertebrae (short-term and long-term) and left femurs (long-term only) were loaded to failure in compression and four-point bending, respectively, using an MTS 858 Mini-Bionix servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN, USA). All specimens were kept hydrated in lactated Ringer's solution (LRS) prior to mechanical testing. The vertebral body was vertically aligned along its loading axis with an alignment pin (attached to lower platen and extending

through the spinal column) and compressed to failure at a displacement rate of 0.05 mm/s. For four-point bending, the posterior surface of the femur was oriented in tension and the mid-diaphysis was loaded to failure at a displacement rate of 0.5 mm/s. Force and vertical displacements were continuously recorded throughout each test by a 50-lb load cell (Sensotec, Columbus, OH, USA) and an external linear variable differential transducer (LVDT; Lucas Schavitts, Hampton, VA, USA), respectively. A custom-developed LABVIEW program was used to calculate the mechanical properties for both tissues.

Statistics

Regional variations in bone architecture and biomechanics among the different treatments were determined using two-way ANOVA (GraphPad Prism 6.0; GraphPad Software, Inc., La Jolla, CA, USA) for each genotype independently. Values of $p<0.05$ were considered significant, and significance is expressed on each plot for independent effects of PAM (vertical arrow) and SclAb (horizontal arrow) within each genotype. Data are reported through dumbbell dot plots that display group means of PAM treatment (white markers) and PAM + SclAb treatment (solid markers) for both Brtl/+ (blue and circles) and WT (black and squares). Standard deviations are shown as shadows behind each marker.

Results

PAM and SclAb contribute distinct gains in distal femoral trabecular number and thickness following a single cycle of combination therapy

At the metaphyseal band site, where BP induces the greatest trabecular retention, PAM monotherapy induced a significant dose-dependent preservation of Tb.N at 0.3 mg/kg (Brtl/+ 3.297

± 1.796/mm; WT 5.677 ± 1.446/mm) and 0.625 mg/kg (Brtl/+ 4.648 ± 1.471/mm; WT 6.225 ± 1.675/mm) compared to vehicle treated control (Brtl/+ 1.905 ± 0.746/mm; WT 3.343 ± 0.574/mm;

Figure 3.2, Table 3.1). Although PAM solely acted to stabilize Tb.N, SclAb monotherapy induced significant gains in both Tb.Th (Brtl/+ 0.049 ± 0.004 mm; WT 0.059 ± 0.007 mm) and Tb.N (Brtl/+ 3.143 ± 0.574 mm; WT 5.272 ± 1.349 mm) compared to untreated control (Brtl/+ 0.035 ± 0.005 mm, 1.905 ± 0.746/mm; WT 0.037 ± 0.003 mm, 3.343 ± 0.574/mm). When PAM and SclAb were administered together, PAM continued to show no effect on Tb.Th, while SclAb triggered consistent thickness gains of 0.049 ± 0.008 mm for Brtl/+ and 0.067 ± 0.013 mm for WT across PAM doses. Interestingly, SclAb alone induced retention of Tb.N comparable to that of 0.3 mg/kg PAM alone (3.143 ± 1.133/mm versus 3.297 ± 1.796/mm). Together, combining SclAb and PAM during a single cycle of combination therapy contributed to a maximum increase in bone volume fraction of 0.263 ± 0.069 for Brtl/+ and 0.443 ± 0.130 for WT over untreated control (Brtl/+ 0.065 ± 0.027; WT 0.124 ± 0.025). Comparing regions proximal and distal to metaphyseal band, we noted that these effects were isolated to the site of concurrent drug administration (see Figure 3.3 and Table 3.2). Trabecular bone distal to BP site showed minimal effect of BP as Tb.N was not significantly different from placebo. However, SclAb induced consistent gains in Tb.Th and Tb.N as monotherapy. At the proximal-most sites formed prior to BP intervention, BP effect was less evident, while significant gains in Tb.Th and BV/TV were still observed with SclAb.

Multiple cycles help stabilize bone mass gains from combination treatment

Following two cycles of combination therapy (Figure 3.4A, Table 3.3), in trabecular regions extending across the entire metaphysis, PAM continued to show no effect on Tb.Th, acting solely to further stabilize Tb.N at 0.3 mg/kg (Brtl/+ 3.813 ± 0.856/mm; WT 4.557 ± 0.624/mm) and 0.625

mg/kg (Brtl/+ 4.329 ± 0.863/mm; WT 5.128 ± 0.687/mm) compared to untreated controls (Brtl/+ 2.929 ± 0.753/mm; WT 4.318 ± 1.131/mm). SclAb continued to induce gains in Tb.Th (Brtl/+ 0.050 ± 0.004 mm; WT 0.057 ± 0.006 mm), as well as a significant preservation of Tb.N (Brtl/+ 4.899 ± 0.595/mm; WT 5.796 ± 0.823/mm) across all PAM doses. As a result of these effects, maximum gains in bone volume fraction of 0.262 ± 0.057 for Brtl/+ and 0.362 ± 0.071 for WT were observed due to cumulative effects from both drugs compared to untreated control (Brtl/+ 0.107 ± 0.039; WT 0.189 ± 0.067). To further explore the effect of dose and time, the entire metaphyseal trabecular bone ROI was subdivided into two separate proximal and distal regions. The proximal-most region represented cycle 1 (Figure 3.4B, Table 3.3), and represents the fate of the band explored in Figure 3.2 following a second cycle of therapy. During a typical course of therapy, BP will interfere with the growth-associated trabecular modeling that would normally cause trabeculae to gradually disappear during growth, thus stabilizing trabecular number over time. Here (Figure 3.4B, Table 3.3), Tb.N of PAM-alone animals drops below levels shown in Figure 3.2, confirming that the low dose of PAM was insufficient to stabilize bone mass long-term. Although the low dose of PAM was sufficient to restore Brtl/+ Tb.N to WT levels after the first cycle, the higher dose was required for the rescue effects to sustain throughout the second cycle. The distal-most region chosen represented cycle 2 (Figure 3.4C, Table 3.3), and shows the retention band induced by the second PAM administration in the presence or absence of SclAb. Here, progressive gains in Tb.N are apparent, and Tb.Th gains in response to SclAb following cycle 2 (Brtl/+ 29%; WT 28%) were substantially less than those observed in the proximal region of interest (Brtl/+ 53%; WT 53%), reflecting either the shorter treatment duration for this site, or a saturation effect of SclAb on trabecular thickness.

Multiple treatments of PAM and SclAb significantly slows the turnover of primary spongiosa

To assess the fate of bone formed during a single treatment cycle (Figure 3.5A), calcein (green) was administered at the beginning of PAM (or control) and alizarin (red) at the end of SclAb (or control) treatment. Using this labeling scheme, bone formed and retained following PAM is shown in green, while bone formed subsequent to PAM is green. During growth, untreated Brtl/+ control showed a significant loss of primary trabeculae, reflected by total absence of green trabecular label (Figure 3.5A, top left). PAM alone increased preservation of trabecular bone shown by retained spicules of calcein label (Figure 3.5A, top right). In contrast SclAb alone (Figure 3.5A, bottom left) exhibited retention of calcein label with adjacent alizarin-labeled bone, indicating trabecular thickening on preserved primary spongiosa. Combined, SclAb and PAM induced an additive bone mass response at the site of concurrent drug administration (Figure 3.5A, bottom right). These patterns of trabecular preservation and thickening amplify under the influence of two cycles of therapy (Figure 3.5B) as gains expand further down the proximal femur compared to gains seen after one cycle (Figure 3.5A). Significant preservation of second-cycle Tb.N is apparent from alizarin-labeled trabeculae in PAM-treated femurs (Figure 3.5B, top right), whereas SclAb alone led to both retention of trabeculae and thickening, indicated by alizarin-labeled trabeculae with abundant calcein label (Figure 3.5B, bottom left). Robust gains in trabeculae and thickness are appreciated when both drugs were given together in multiple cycles as seen through retained alizarin-labeled trabeculae with a calcein-labeled thickness (Figure 3.5B, bottom right).

Multiple cycles of combination therapy induce synergistic gains in vertebral trabecular bone mass

Microstructural analysis of the lumbar vertebra demonstrated similar findings, albeit to a lesser extent, as seen in the femoral metaphysis, confirming consistency of effects at axial and

appendicular sites. Under the influence of a single treatment cycle (Figure 3.6A, Table 3.4), PAM monotherapy significantly stabilized Tb.N at 0.3 mg/kg (Brtl/+ 6.137 ± 0.536/mm; WT 6.573 ± 0.459/mm) and 0.625 mg/kg (Brtl/+ 6.376 ± 0.569/mm; WT 6.886 ± 0.363/mm) over placebo control (Brtl/+ 5.787 ± 0.300/mm; WT 6.130 ± 0.271/mm), with no effect on Tb.Th. SclAb monotherapy produced gains in both Tb.N (Brtl/+ 6.208 ± 0.420/mm; WT 6.816 ± 0.376/mm) and Tb.Th (Brtl/+ 0.046 ± 0.002 mm; WT 0.053 ± 0.004 mm). As found in the femur, when SclAb was administered with PAM, little additional gains were observed in Tb.N. Higher doses of PAM were required to see additional trabecular thickening in Brtl/+, while WT showed an average 0.055 mm increase in Tb.Th with the two drugs combined. Despite these observations during a single cycle of combination therapy, overall gains in BV/TV were comparable whether drugs were given combined (Brtl/+ 0.298 ± 0.043; WT 0.388 ± 0.029) or SclAb alone (Brtl/+ 0.289 ± 0.032; WT 0.365 ± 0.040). Lumbar vertebrae benefited strongly from two treatment cycles (Figure 3.6B), triggering a maximum synergistic response on BV/TV (Brtl/+ 0.421 ± 0.053; WT 0.504 ± 0.053) over placebo (Brtl/+ 0.235 ± 0.026; WT 0.293 ± 0.037) compared to 0.625 mg/kg PAM (Brtl/+ 0.275 ± 0.046; WT 0.315 ± 0.033) and SclAb (Brtl/+ 0.339 ± 0.043; WT 0.452 ± 0.065) monotherapy. PAM continued to increase bone mass through Tb.N at 0.3 mg/kg (Brtl/+ 6.002 ± 0.444/mm; WT 6.487 ± 0.346/mm) and at 0.625 mg/kg (Brtl/+ 6.300 ± 0.614/mm; WT 6.838 ± 0.340/mm) with no effect on Tb.Th. SclAb induced strong gains in both Tb.N (Brtl/+ 6.867/mm; WT 6.889/mm) and Tb.Th (Brtl/+ 0.058 ± 0.004 mm; WT 0.070 ± 0.007 mm) across all PAM dosages, contributing substantially to the overall synergistic gains in lumbar vertebrae trabecular bone mass.

Substantial preservation of Tb.N and increased Tb.Th in vertebral body

To evaluate the response of newly formed bone under the effects of a single treatment cycle (Figure 3.7A), calcein (green) was administered prior to PAM treatment and alizarin (red) after SclAb treatment. Brtl/+ control showed few primary trabeculae labeled with calcein, which was stabilized with a single dose of PAM. SclAb showed preservation of calcein labeled bone with alizarin labeled perimeters suggesting trabecular thickening. These observations confirm the antiresorptive and anabolic effects of both treatments, which led to further additive gains in bone mass. Newly formed bone under the effects of multiple treatment cycles (Figure 3.7B), was labeled with calcein (green) at the beginning of treatment, alizarin (red) between cyclic treatments and again with calcein (green) at the end of treatment. Labeling of control samples showed considerable loss of primary trabeculae. PAM alone promoted the retention of primary trabeculae, noted by considerable alizarin-labeled bone adjacent to the growth plates. SclAb alone triggered both trabecular retention and thickness gains. Combination of both drugs administered cyclically induced a robust trabecular response which extended to the periosteal bone surface.

PAM intervention does not interfere with SclAb ability to improve femoral rigidity

PAM effects were isolated to the lumbar vertebrae and femoral trabecular bone, with no apparent effects on femoral diaphyseal cortical bone structure or biomechanical properties. Rather, gains in cortical thickness were solely attributed to SclAb because our administered PAM treatment used doses significantly lower than those used in human studies. After a single cycle of combination therapy (Figure 3.8A, Table 3.5), SclAb monotherapy induced greater gains in femoral C.Th (Brtl/+ 0.182 ± 0.008 mm; WT 0.234 ± 0.009 mm) than when combined with 0.3 mg/kg PAM (Brtl/+ 0.171 ± 0.017 mm; WT 0.234 ± 0.009mm) or 0.625 mg/kg PAM (Brtl/+ 0.176 ± 0.012 mm; WT 0.216 ± 0.028mm) compared to placebo (Brtl/+ 0.152 ± 0.016 mm; WT 0.192 ± 0.022

mm). However, this effect was transient, because after two cycles (Figure 3.8B), consistent C.Th gains of 0.231 ± 0.023 mm in Brtl/+ and 0.277 ± 0.023 mm in WT were observed across all PAM doses. As a result of these C.Th changes, SclAb consistently improved femoral rigidity through gains in stiffness (Brtl/+ 231.768 ± 44.278N/mm; WT 272.632 ± 57.425N/mm) and ultimate load (Brtl/+ 35.379 ± 6.741 N; WT 47.816 ± 9.937 N) across all PAM doses, driven by gains in bending moment (Brtl/+ 0.158 ± 0.045 mm^4; WT 0.207 ± 0.028 mm^4) and cross-sectional area (Brtl/+ 1.919 ± 0.200 mm^2; WT 2.234 ± 0.267mm^2) as seen in Figure 3.8C.

PAM and SclAb synergistically improve Brtl/+ vertebral stiffness over monotherapy effects

Similar to the cortical effects seen in the femur, C.Th gains in the vertebral body were influenced by SclAb, not PAM. A single cycle of combination therapy (Figure 3.9A, Table 3.6) resulted in lower gains in C.Th at 0.3 mg/kg (Brtl/+ 0.086 ± 0.007 mm; WT 0.102 ± 0.006 mm) and 0.625 mg/kg (Brtl/+ 0.088 ± 0.007 mm; WT 0.102 ± 0.007 mm) than SclAb monotherapy (Brtl/+ 0.091 ± 0.005 mm; WT 0.100 ± 0.005 mm). However, with multiple cycles (Figure 3.9B), greater gains in C.Th were achieved at 0.3 mg/kg (Brtl/+ 0.090 ± 0.006 mm; WT 0.103 ± 0.010 mm) and 0.625 mg/kg (Brtl/+ 0.093 ± 0.009 mm; WT 0.106 ± 0.008 mm). Functionally, over a single cycle of therapy (Figure 3.9C), PAM dose-dependently improved vertebral stiffness and SclAb amplified this response (Brtl/+ 29%, WT 32%). Improvements in ultimate load were attributed to SclAb with gains of 48% in Brtl/+ and 58% in WT. Over two cycles of combination treatment (Figure 3.9D), maximum gains in vertebral stiffness were observed when both PAM (Brtl/+ 75.650 ± 8.031 N/mm; WT 119.729 ± 24.514 N/mm) and SclAb (Brtl/+ 81.307 ± 15.531 N/mm; WT 136.688 ± 16.367 N/mm) were administered alone. When administered together in sequential cycles, WT showed maximum additive gains (156.340 ± 28.962 N/mm) compared to each treatment alone.

Remarkably, a synergistic response was observed in Brtl/+, with a maximum stiffness increase of 130.945 ± 25.920 N/mm compared to placebo control (46.974 ± 20.181 N/mm).

Discussion

The results in this study show Brtl/+ mice with a genetic knock-in for moderately severe Type IV OI responded favorably to repeated cycles of a single dose of antiresorptive PAM in combination with anabolic SclAb. The immediate effects of this combination therapy showed that both interventions stimulated gains in bone mass through different means, leading to overall improvements in biomechanical function. Although a preservation of Tb.N was observed through PAM, SclAb led to gains in both Tb.N and Tb.Th, as well as a significant cortical bone response. When this combination therapy was administered twice cyclically, the resulting gains in trabecular bone mass were dependent on anatomic site; cumulative for long bones and synergistic for the vertebral body. These findings may have a strong clinical utility to increase bone mass in OI patients—particularly those with excessive high bone turnover leading to severe trabecular osteopenia that might make them more resistant to anabolic therapy alone.

Antiresorptive BPs continue to be the most common intervention used in pediatric OI. Numerous studies have shown a favorable bone response to BP therapy in both preclinical studies using OI mouse models [24, 28, 174, 184] and clinical studies of children with OI. [20, 22, 24-26, 167, 185] By their antiresorptive activity, BPs have been proposed to decrease the high bone turnover characteristic of OI, increase metaphyseal bone mass by increasing trabecular number, and increase cortical bone mass by inhibiting endosteal resorption. However, concern for long half-life,[186] microdamage accumulation,[187] and delayed healing [188] have led to attempts to

minimize treatment dose. [175] Recently, SclAb has gained interest as a promising anabolic approach for the treatment of OI. In contrast to BP, SclAb has been shown to increase trabecular bone mass in OI models through trabecular thickening, and increase cortical bone mass through periosteal apposition. [47, 48, 50] Furthermore, in a growing model, periosteal apposition will occur as a result of growth, which contributes to further bone mass gains because PAM has not been shown to affect non-remodeling surfaces. Therefore, we hypothesized that a combination of these two agents may lead to additive if not synergistic structural and functional gains. When given together, we observed that BPs induce retention of primary trabeculae, which can serve as a substrate for the subsequent anabolic response of SclAb, whereas cortical bone mass gains resulted from SclAb alone.

Combination effects have been explored with BP following SclAb in order to preserve gains in bone mass following cessation of drug clinically [189] and in OI models. [53] Alternatively, SclAb following BP has been explored using ovariectomized rats in which SclAb induced gains in bone mass regardless of prior BP exposure. [190] This study was performed in aged, osteoporotic rats, which have considerably different growth plate dynamics than in the present study. In another OI mouse model, SclAb was combined with zoledronic acid,[95] where no synergistic effects were observed. Rather, the treatment effect from zoledronic acid alone led to >300% gains in proximal tibial trabecular bone volume fraction, with no additional gains observed when zoledronic acid was combined with SclAb. In the present combination study, we likely avoided this saturation response with lower doses of BP, triggering more modest trabecular preservation, allowing SclAb to synergistically increase bone mass by increasing trabecular thickness.

The present study differs from these other combination therapy studies because we aimed to explore low doses of PAM which, if given alone, would be insufficient to generate long-term

preservation of trabecular bone. Indeed, when tracking the fate of the trabecular bone generated by the first cycle of PAM, we observed a lack of long-term antiresorptive effects, particularly in the vertebrae. Yet when administered together with SclAb, both drugs combined led to cumulative gains on bone mass. At the site of concurrent treatment, PAM induced a sclerotic metaphyseal band consistent with previous observations of BP treatment stabilizing primary spongiosa near the growth plate. [191] Treatment with PAM alone showed a significant effect on bone volume fraction for both Brtl/+ and WT when compared to untreated controls, solely due to a significant dose-dependent preservation of Tb.N with no concurrent effect on Tb.Th. Similar gains in bone volume were triggered with SclAb monotherapy; however, these were attributed to an effect on both Tb.N and Tb.Th. Gains in Tb.Th likely resulted from a direct anabolic effect on existing trabecular bone. However, gains in Tb.N could have resulted from either stabilization of thin trabeculae destined for remodeling via increased bone formation on those surfaces, or through a mild antiresorptive effect, because SclAb has been shown to have both anabolic and anticatabolic actions. [192] Indeed, SclAb alone preserved Tb.N to levels equivalent to low-dose PAM, suggesting a similar potency of antiresorptive action between the two drugs. When given together, extended gains in trabecular bone mass were attributed to SclAb-induced trabecular thickening at sites formed following PAM exposure and retention of trabecular number. These observations were confirmed through histomorphometry. Observations at sites distal and proximal to the metaphyseal band helped verify that PAM bone response was constrained to its induced sclerotic band, while SclAb led to trabecular thickening away from these sites.

The same trabecular response was observed in the vertebral body for both Brtl/+ and WT, to a slightly greater extent, as was observed in the femoral metaphysis. These findings are consistent with other BP studies that show a variable BP binding and bone density response between axial

versus appendicular, and cortical versus trabecular sites. [181, 193] Following two cycles of combination therapy, a synergistic effect on bone mass was observed in the vertebral body that was not observed in the femoral metaphysis, which instead showed an additive effect of the two interventions. Histomorphometry of two cycles of combination therapy demonstrated thickened bands of retained primary spongiosa emerging from both growth plates of the vertebra consistent observations in other pediatric BP studies. [183, 194, 195]

In addition to trabecular effects, SclAb induced an anabolic response in femoral and vertebral cortical bone, because gains observed were solely attributed to SclAb, even when administered in combination with PAM. This increase in cortical thickness correlates with previous findings where growing Brtl/+ mice treated with SclAb showed cortical thickness gains in the femoral midshaft over a similar treatment duration. [47] Furthermore, progressive gains in cortical thickness were observed comparing one versus two cycles of SclAb monotherapy. Although clinical studies of children with OI have shown slight changes in cortical bone from PAM treatment, [174, 196-198] in the present study, PAM had no effect on cortical thickness, likely due to the low treatment duration and doses used in the present study compared to those in clinical trials. Low doses of PAM were deliberately chosen for this study in order to minimize the antiresorptive effect and not mask the potential combination effects hypothesized from both drugs together.

Two cycles of PAM monotherapy showed no effect on femoral stiffness or load in either Brtl/+ and WT. SclAb, on the other hand, improved both stiffness and load as a result of the strong anabolic effect on cortical bone. When both drugs were administered concurrently, SclAb continued to be the sole contributor to these improvements in whole-bone mechanical properties.

Similarly, in the vertebral body, Brtl/+ ultimate load was only improved by SclAb, yet both PAM and SclAb led to strong gains in vertebral stiffness. In fact, following two cycles of combined treatment, Brtl/+ stiffness improved synergistically when compared to combined effects from monotherapy of either drug. This finding may have important implications for treatment of spinal deformities in OI. Deformation of the spine arises from both anatomical and functional factors resulting from the OI phenotype. Depending on the severity of the deformity or fracture frequency, prophylactic interventions like BP treatment can potentially reduce fracture incidence. Combining SclAb with PAM stabilized vertebral trabecular bone resulting in significant gains in vertebral stiffness. SclAb alone improved maximum load, likely due to additional gains in cortical thickness that were not evident following PAM. These findings confirm other studies that have shown that C.Th is a primary determinant feature for compressive strength of the whole vertebral body. [199-201]

This study has several limitations. Because OI is a disease of many mutations, the results of this study may not extend to all types of OI. Additionally, the Brtl/+ mouse fragility phenotype is of moderate severity, and the mouse does not suffer spontaneous fractures. In addition, the study was only performed in male mice and it is now well established that gender can affect the outcome of drug treatments. Furthermore, BP action when given after closure of the growth plate during skeletal maturity will not yield the same findings as observed in this study because turnover of primary spongiosa will be nonexistent. In many of our outcomes, treatment with our lowest PAM dose alone improved bone mass to WT control levels, effectively rescuing the phenotype, while our higher PAM dose induced gains in bone mass greater than WT baseline. Although our goal was not to directly titrate our treatment outcomes to match untreated WT levels, we believe that

further adjustment of treatment doses and/or duration will vary depending on clinical severity to best represent a “rescue” response.

SclAb has been proposed as a novel anabolic intervention to treat the low bone mass and fragility phenotype present in patients with OI. The results in this study extend our previous observations of single-drug therapy in Brtl/+ [47, 174] and show the benefits of combining SclAb with antiresorptive treatment during growth. In severe cases of OI, where extreme low bone mass may result from excessive resorption of primary trabeculae during endochondral growth, SclAb alone might not be enough to significantly improve trabecular bone volume because of lack of bone upon which to exert an anabolic action. Thus, in cases of extreme low bone mass, concurrent antiresorptive and anabolic therapy might further improve bone formation outcomes. Importantly, these results suggest that modest doses of BP, which would otherwise not induce a sustained therapeutic effect, may be sufficient to preserve trabecular architecture enough to permit an anabolic action of SclAb on bone which would otherwise be remodeled. Together, these preclinical results support the scientific premise that antiresorptive and anabolic combination therapy during early stages of skeletal growth can help induce greater gains in bone mass in OI than either drug alone. The present data provides key preclinical results to support a treatment plan to maximize combination therapy in OI, or other diseases associated with low bone mass during pediatric growth and development.

Figures

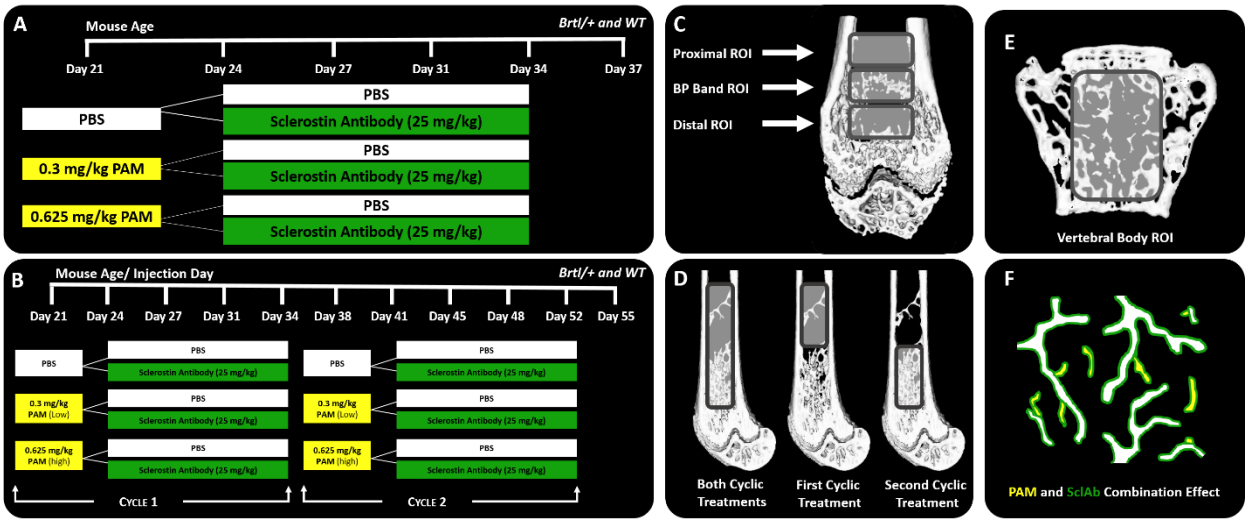

Figure 3.1: *Schematic of short and long-term treatment timelines and ROI placement.* Combination therapy to assess the effects after (A) a single cycle and (B) concurrent cycles of combination therapy. Region of interest placement in distal femoral metaphysis to analyze the (C) short-term, (D) long-term, and (E) vertebral trabecular bone effects of combination therapy. (F) Proposed mechanism of action for combination therapy.

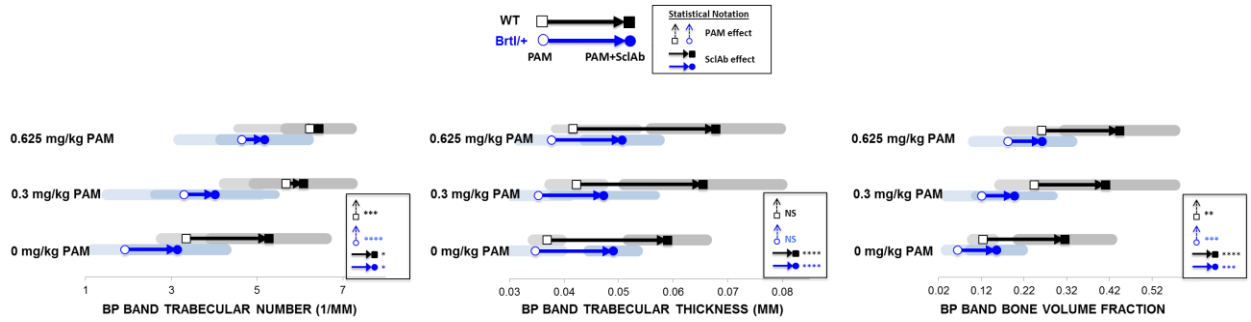

Figure 3.2: *Trabecular response at the femur following single cycle of combination therapy.* µCT analysis at the site of concurrent drug administration showed that PAM induced a significant dose-dependent increase in Tb.N whereas SclAb contributed to significant gains in both Tb.N and Tb.Th. Together these independent gains led to an additive gain in bone volume fraction with increasing PAM doses. Results of two-way ANOVA factors: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

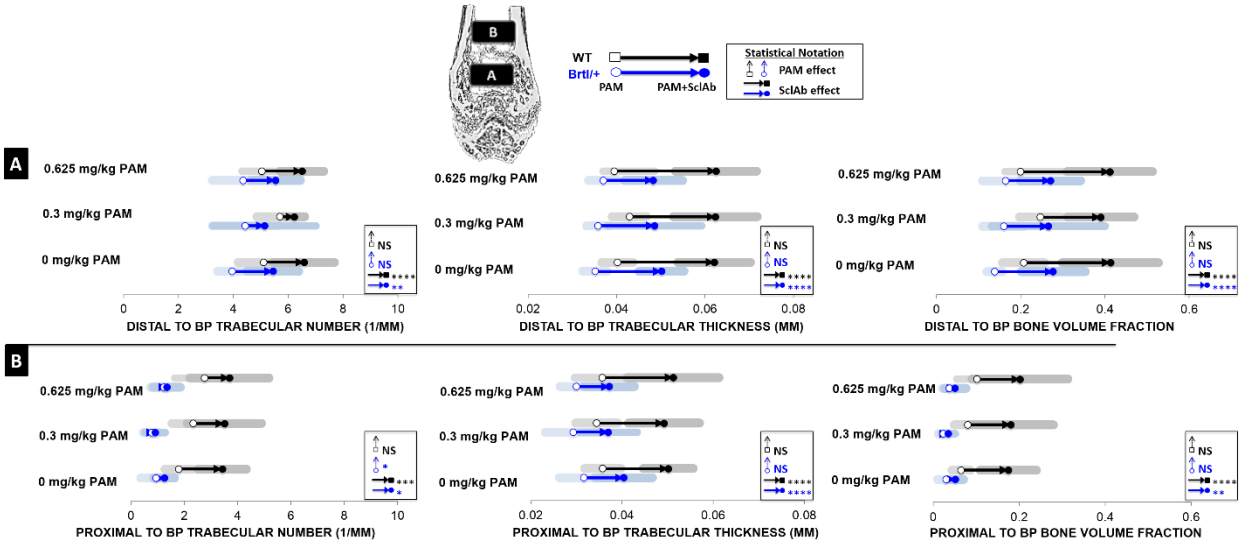

Figure 3.3: *Isolated effects proximal and distal to site of concurrent drug administration.* A) Sites of bone formed following PAM cessation showed that gains in bone volume fraction were attributed solely to SclAb-induced trabecular thickening, with an average increase of 40% for Brtl/+ and 55% for WT across PAM doses. Gains in TbN were attributed solely to SclAb, not PAM, reflecting treatment site specificity and a mild anti-resorptive effect of the drug. B) Region proximal to metaphyseal band, representing trabecular bone formed prior to BP injection but under the influence of SclAb, showed a significant effect on bone volume primarily due to an average trabecular thickening of 21% for Brtl/+ and 40% for WT.

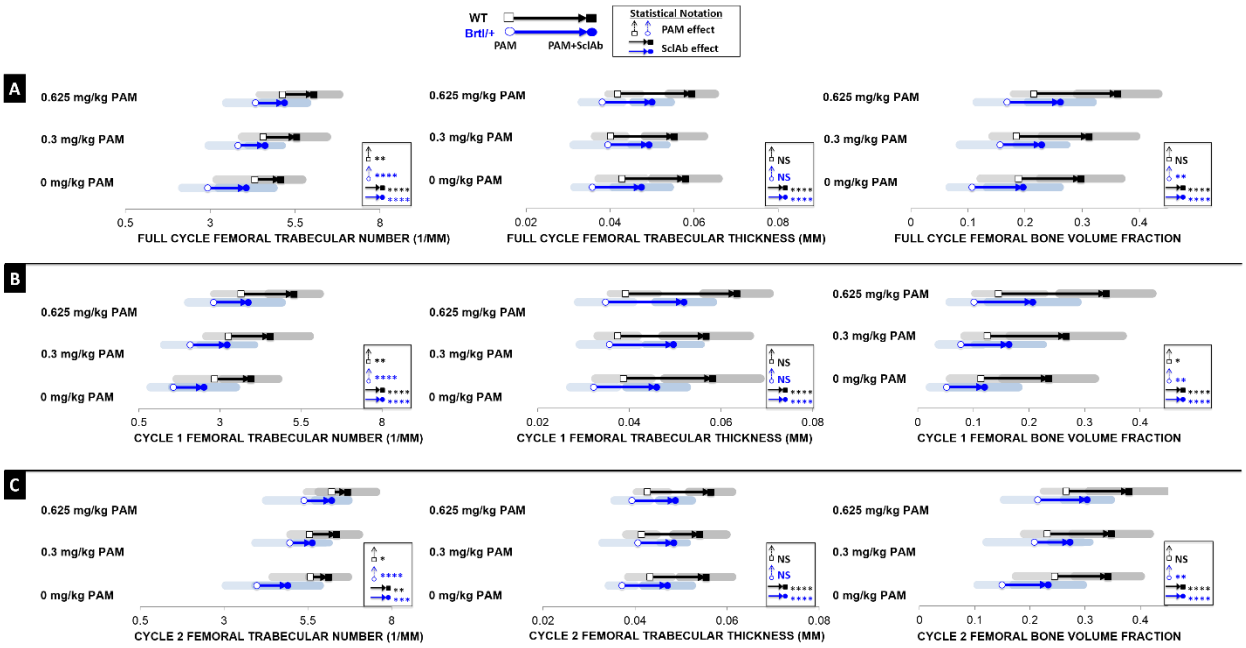

Figure 3.4: *Trabecular response at the femur following two cycles of combination therapy.* (A) Entire metaphyseal trabecular bone ROI following two cycles of combination therapy showed that PAM continued to influence Tb.N while inducing no effects on Tb.Th. SclAb, however, induced gains in both Tb.Th and Tb.N. Each cyclic treatment was separately analyzed by subdividing the entire metaphyseal trabecular bone ROI into (B) cycle 1 and (C) cycle 2. Isolating the effects of each administered cycle confirmed that these low doses of PAM were weak in sustaining long-term trabeculae preservation. Results of two-way ANOVA factors: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

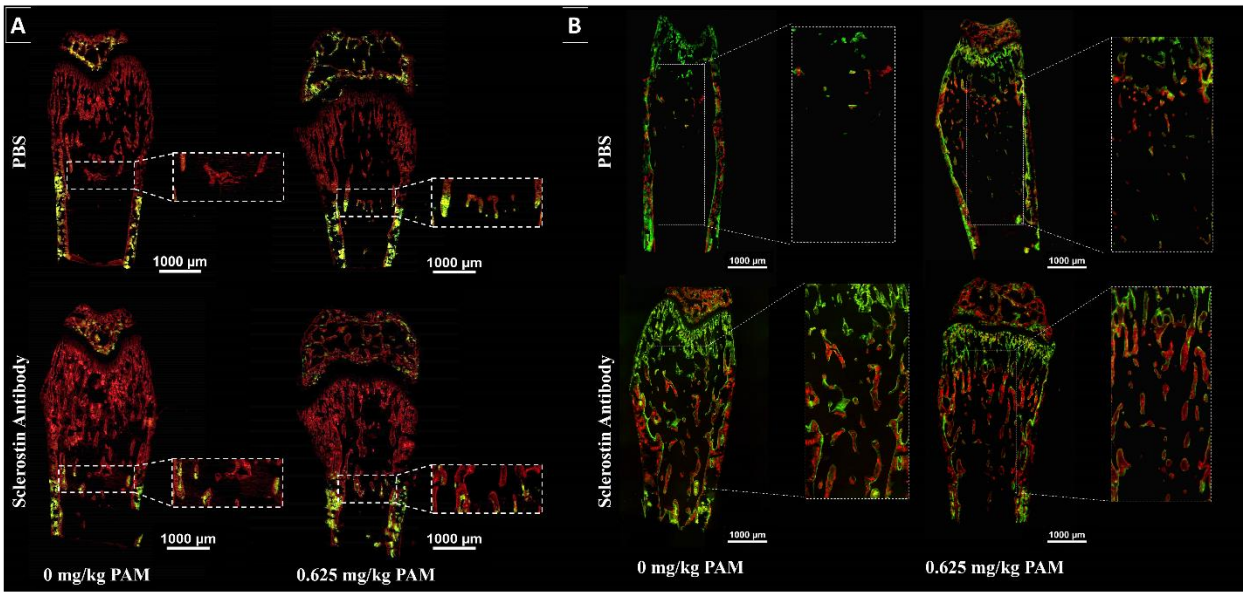

Figure 3.5: *Metaphyseal bone formation activity mapped with calcein and alizarin following a single and concurrent cycles of combination therapy*. Histological sections of Brtl/+ metaphysis revealed short-term (A) and long-term (B) bone formation activity from a single and concurrent cycles of combination therapy, respectively. (A) At the BP-induced retention region, higher dosages of PAM were associated with greater primary trabeculae retention (yellow), while SclAb predominantly increased thickness (red). (B) Consecutive cycles of combination therapy showed a greater robust effect on bone mass with higher dosages of PAM. Calcein (green) was administered at the beginning and end of concurrent treatment cycles while alizarin (red) was administered prior to the second cyclic treatment. Sections visualized represent n = 4 per group.

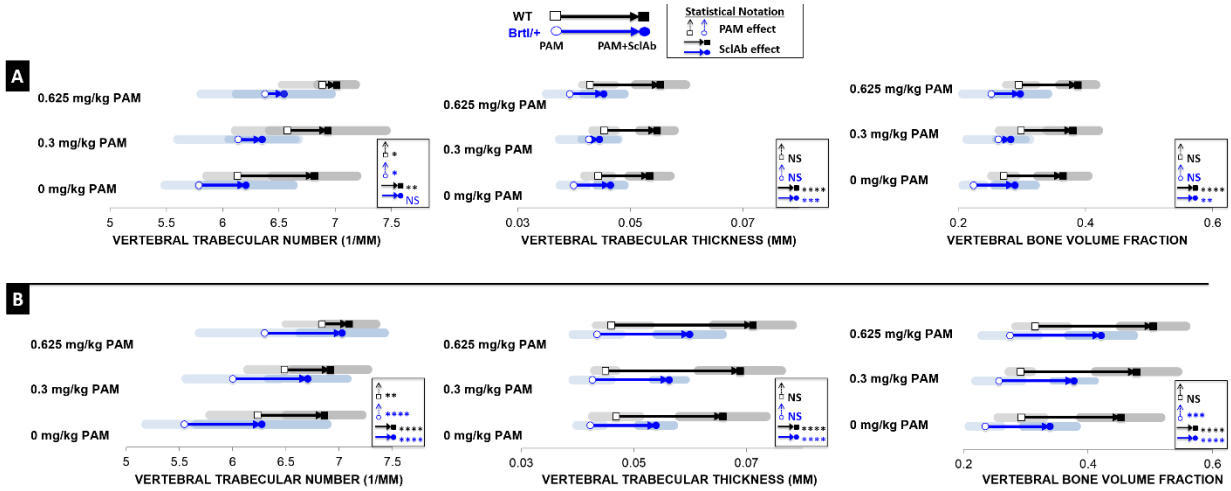

Figure 3.6: *Trabecular response in the vertebra following single cycle of combination therapy.* (A) Microstructural properties of the vertebral trabecular bone reveal overall preservation of Tb.N with PAM and increased Tb.Th with SclAb after a single cycle of combination therapy. (B) With multiple treatment cycles, the response on Tb.N and Tb.Th was highly amplified leading to synergistic gains on bone mass. Results of two-way ANOVA factors: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

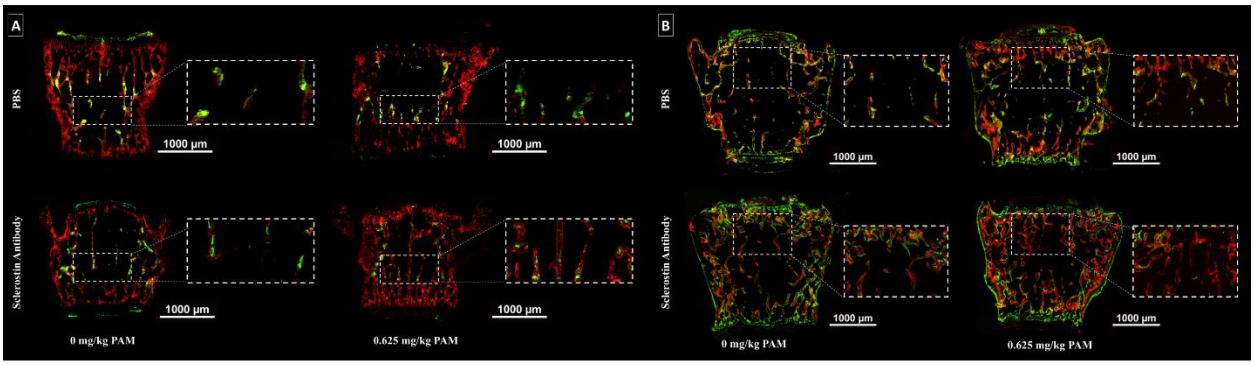

Figure 3.7: *Vertebral bone formation activity mapped with calcein and alizarin following a single and concurrent cycles of combination therapy.* Histological coronal sections of Brtl/+ revealed bone formation activity from a single (A) and multiple (B) cycles of combination therapy. (A) Under a single treatment cycle, higher dosages of PAM were associated to greater primary trabeculae retention (green), while SclAb predominantly increased thickness and connectivity between growth plates (red). (B) Multiple treatment cycles showed an evident trabecular retention response from both PAM and SclAb; however, SclAb also induced gains in trabecular thickness (green). Together a robust trabecular response was triggered leading to uniform trabecular bone distribution between growth plates. Sections visualized represent n = 4 per group.

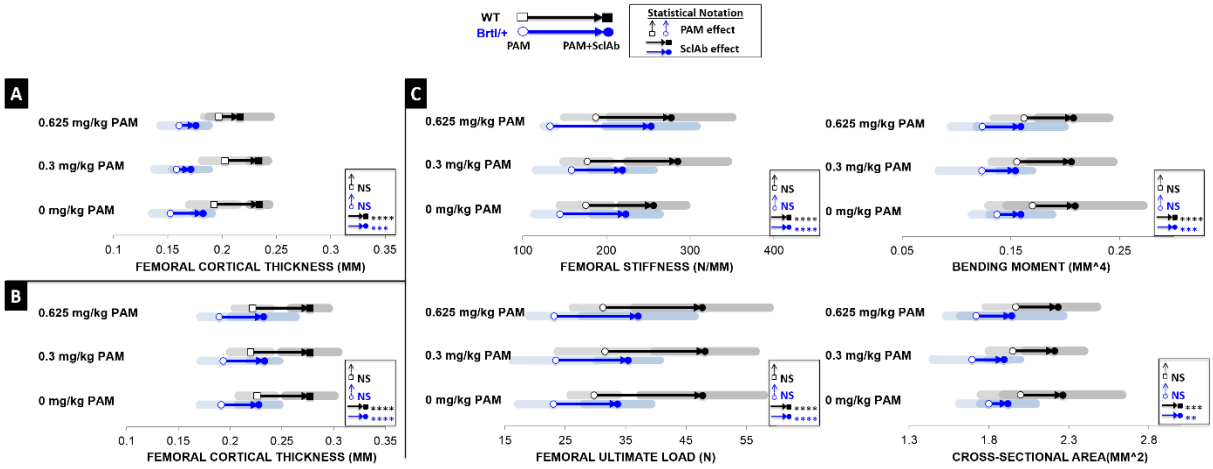

Figure 3.8: *Effects on femoral mechanical and cortical properties from a single and concurrent cycles of combination therapy.* (A) Femoral cortical analysis revealed SclAb influence on C.Th varies in the presence of PAM after a single treatment cycle. (B) Following subsequent cycles of combination therapy, C.Th nearly doubled solely in response to SclAb. (C) Functionally, multiple cycles PAM and SclAb led to additive gains in femoral stiffness and ultimate load with progressive PAM doses. Results of two-way ANOVA factors: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

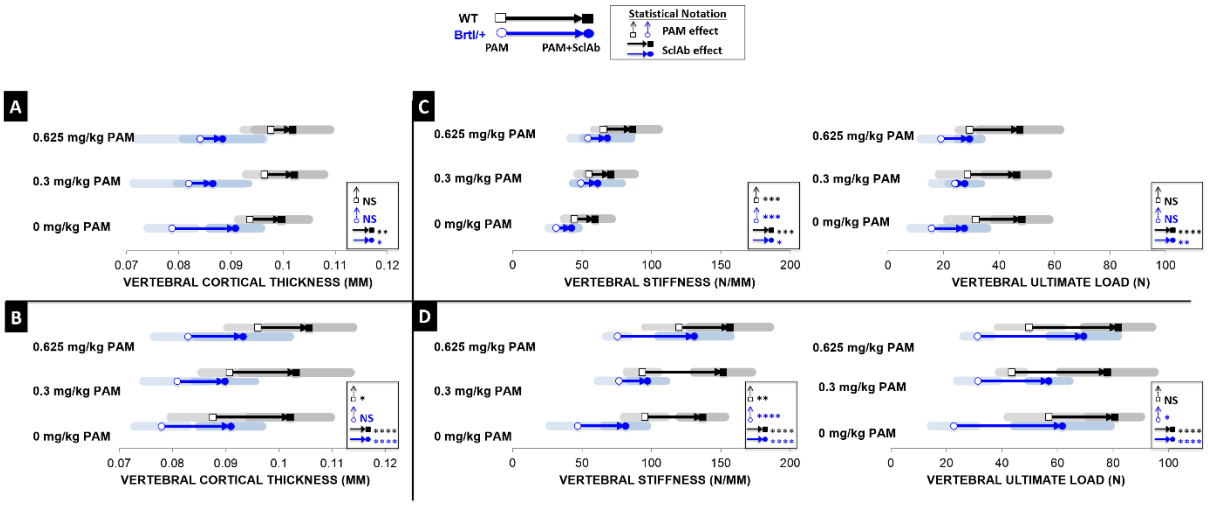

Figure 3.9: *Effects on vertebral mechanical and cortical properties from a single and concurrent cycles of combination therapy.* (A) Vertebral cortical analysis revealed that after a single cycle, SclAb triggered greater gains in C.Th than when combined with PAM. (B) Following two cycles, however, consistent gains in C.Th were observed across all PAM dosages. (C) Functionally, following a single combination cycle, PAM effect on trabecular preservation helped improve vertebral stiffness while SclAb amplified these effects. Significant improvements in ultimate load were solely attributed to SclAb. (D) Both drugs improved ultimate load through an additive response, however, induced a synergistic effect on vertebral stiffness following multiple cycles of combination treatment. Results of two-way ANOVA factors: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Tables

Table 3.1: *Trabecular response at the femur following single cycle of combination therapy.* MicroCT analysis at the site of concurrent drug administration showed that PAM induced a significant dose-dependent increase in TbN while SclAb contributed to significant gains in both TbN and TbTh. Together these independent gains led to an additive gain in bone volume fraction with increasing PAM doses. The mean and standard deviation (mean ± SD) of each bone parameter is reported.

	Combination Treatment		BP Band TbN (1/mm)	BP Band TbTh (mm)	BP Band BV/TV
HET	0 mg/kg PAM	PBS	1.905 ± 0.746	0.035 ± 0.005	0.065 ± 0.027
		SclAb	3.143 ± 1.133	0.049 ± 0.004	0.156 ± 0.061
	0.3 mg/kg PAM	PBS	3.297 ± 1.796	0.035 ± 0.006	0.121 ± 0.083
		SclAb	4.017 ± 1.409	0.047 ± 0.009	0.198 ± 0.091
	0.625 mg/kg PAM	PBS	4.648 ± 1.471	0.038 ± 0.006	0.182 ± 0.080
		SclAb	5.176 ± 1.023	0.051 ± 0.007	0.263 ± 0.069
WT	0 mg/kg PAM	PBS	3.343 ± 0.574	0.037 ± 0.003	0.124 ± 0.025
		SclAb	5.272 ± 1.349	0.059 ± 0.007	0.316 ± 0.109
	0.3 mg/kg PAM	PBS	5.677 ± 1.446	0.042 ± 0.005	0.244 ± 0.080
		SclAb	6.077 ± 1.143	0.065 ± 0.014	0.411 ± 0.163
	0.625 mg/kg PAM	PBS	6.225 ± 1.675	0.042 ± 0.003	0.261 ± 0.080
		SclAb	6.435 ± 0.767	0.068 ± 0.012	0.443 ± 0.130

Table 3.2: *Isolated effects proximal and distal to site of concurrent drug administration.* MicroCT analysis of sites of bone formed following PAM cessation showed that gains in bone volume fraction were attributed solely to SclAb-induced trabecular thickening across all PAM dosages. Gains in TbN were attributed solely to SclAb, not PAM, reflecting treatment site specificity and a mild anti-resorptive effect of the drug. Region proximal to metaphyseal band, representing trabecular bone formed prior to BP injection but under the influence of SclAb, showed a significant effect on bone volume primarily due to an average trabecular thickening.

	Combination Treatment		Proximal to BP Band			Distal to BP Band		
			TbN (1/mm)	TbTh (mm)	BV/TV	TbN (1/mm)	TbTh (mm)	BV/TV
HET	0 mg/kg PAM	PBS	0.929 ± 0.556	0.032 ± 0.005	0.030 ± 0.020	3.954 ± 0.529	0.035 ± 0.003	0.139 ± 0.018
		SclAb	1.251 ± 0.384	0.040 ± 0.006	0.051 ± 0.019	5.447 ± 0.939	0.050 ± 0.005	0.277 ± 0.076
	0.3 mg/kg PAM	PBS	0.762 ± 0.305	0.029 ± 0.006	0.024 ± 0.012	4.428 ± 1.195	0.036 ± 0.003	0.160 ± 0.050
		SclAb	0.900 ± 0.380	0.037 ± 0.006	0.035 ± 0.017	5.142 ± 1.877	0.049 ± 0.011	0.266 ± 0.135
	0.625 mg/kg PAM	PBS	1.221 ± 0.495	0.030 ± 0.004	0.037 ± 0.016	4.355 ± 1.126	0.037 ± 0.003	0.164 ± 0.054
		SclAb	1.342 ± 0.512	0.037 ± 0.006	0.051 ± 0.025	5.542 ± 0.902	0.048 ± 0.007	0.271 ± 0.072
WT	0 mg/kg PAM	PBS	1.784 ± 0.508	0.036 ± 0.004	0.065 ± 0.021	5.108 ± 0.932	0.040 ± 0.004	0.207 ± 0.050
		SclAb	3.415 ± 0.913	0.050 ± 0.005	0.175 ± 0.065	6.577 ± 1.114	0.062 ± 0.008	0.413 ± 0.114
	0.3 mg/kg PAM	PBS	2.321 ± 0.776	0.034 ± 0.005	0.081 ± 0.032	5.700 ± 0.836	0.043 ± 0.004	0.247 ± 0.050
		SclAb	3.502 ± 1.389	0.049 ± 0.008	0.181 ± 0.099	6.223 ± 0.373	0.062 ± 0.009	0.390 ± 0.079
	0.625 mg/kg PAM	PBS	2.753 ± 1.112	0.036 ± 0.006	0.102 ± 0.047	5.038 ± 0.721	0.039 ± 0.003	0.200 ± 0.038
		SclAb	3.699 ± 1.479	0.051 ± 0.010	0.202 ± 0.112	6.504 ± 0.789	0.062 ± 0.009	0.412 ± 0.101

Table 3.3: *Trabecular response at the femur following two cycles of combination therapy* Entire metaphyseal trabecular bone ROI following two cycles of combination therapy showed that PAM continued to influence TbN while inducing no effects on TbTh. SclAb, however, induced gains in both TbTh and TbN. Each cyclic treatment was separately analyzed by subdividing the entire metaphyseal trabecular bone ROI into their respective cycles. Isolating the effects of each administered cycle confirmed that these low doses of PAM were weak in sustaining long-term trabeculae preservation. The mean and standard deviation (mean ± SD) of each bone parameter is reported.ion proximal to metaphyseal band, representing tracular bone formed prior to BP injection but under the influence of SclAb, showed a significant effect on bone volume primarily due to an average trabecular thickening.

	Combination Treatment		Full Cycle TbN (1/mm)	Full Cycle TbTh (mm)	Full Cycle BV/TV	Cycle 1 TbN (1/mm)	Cycle 1 TbTh (mm)	Cycle 1 BV/TV	Cycle 2 TbN (1/mm)	Cycle 2 TbTh (mm)	Cycle 2 BV/TV
HET	0 mg/kg PAM	PBS	2.929 ± 0.753	0.036 ± 0.004	0.107 ± 0.039	1.549 ± 0.691	0.032 ± 0.005	0.052 ± 0.030	3.978 ± 0.922	0.037 ± 0.003	0.150 ± 0.044
		SclAb	4.057 ± 0.812	0.048 ± 0.007	0.197 ± 0.064	2.511 ± 0.990	0.046 ± 0.007	0.120 ± 0.061	4.898 ± 0.963	0.047 ± 0.005	0.233 ± 0.063
	0.3 mg/kg PAM	PBS	3.813 ± 0.856	0.039 ± 0.008	0.156 ± 0.071	2.077 ± 0.796	0.036 ± 0.006	0.077 ± 0.040	4.966 ± 1.032	0.041 ± 0.008	0.208 ± 0.087
		SclAb	4.612 ± 0.516	0.049 ± 0.004	0.229 ± 0.045	3.220 ± 0.853	0.050 ± 0.006	0.164 ± 0.063	5.626 ± 0.517	0.048 ± 0.003	0.273 ± 0.036
	0.625 mg/kg PAM	PBS	4.329 ± 0.863	0.038 ± 0.005	0.168 ± 0.053	2.804 ± 0.784	0.035 ± 0.006	0.101 ± 0.044	5.381 ± 1.124	0.039 ± 0.004	0.214 ± 0.061
		SclAb	5.186 ± 0.674	0.050 ± 0.006	0.262 ± 0.057	3.877 ± 1.037	0.052 ± 0.006	0.207 ± 0.081	6.211 ± 0.499	0.049 ± 0.004	0.304 ± 0.043
WT	0 mg/kg PAM	PBS	4.318 ± 1.131	0.043 ± 0.006	0.189 ± 0.067	2.831 ± 1.161	0.039 ± 0.006	0.114 ± 0.056	5.576 ± 1.127	0.043 ± 0.005	0.245 ± 0.070
		SclAb	5.073 ± 0.636	0.058 ± 0.008	0.297 ± 0.072	3.946 ± .853	0.058 ± 0.011	0.235 ± 0.084	6.112 ± 0.581	0.055 ± 0.006	0.341 ± 0.060
	0.3 mg/kg PAM	PBS	4.557 ± 0.624	0.040 ± 0.004	0.184 ± 0.042	3.264 ± 0.682	0.037 ± 0.004	0.125 ± 0.041	5.556 ± 0.571	0.041 ± 0.003	0.231 ± 0.042
		SclAb	5.544 ± 0.893	0.055 ± 0.007	0.312 ± 0.083	4.545 ± 1.227	0.057 ± 0.010	0.267 ± 0.102	6.358 ± 0.674	0.054 ± 0.006	0.347 ± 0.070
	0.625 mg/kg PAM	PBS	5.128 ± 0.687	0.042 ± 0.002	0.215 ± 0.036	3.649 ± 0.842	0.039 ± 0.003	0.145 ± 0.043	6.210 ± 0.759	0.043 ± 0.002	0.266 ± 0.042
		SclAb	6.048 ± 0.753	0.059 ± 0.006	0.362 ± 0.071	5.286 ± 0.796	0.063 ± 0.007	0.339 ± 0.084	6.677 ± 0.863	0.056 ± 0.005	0.379 ± 0.072

Table 3.4: *Trabecular response in the vertebra following single cycle of combination therapy.*Microstructural properties of the vertebral trabecular bone reveal overall preservation of TbN with PAM and increased TbTh with SclAb after a single cycle of combination therapy. With multiple treatment cycles, the response on TbN and TbTh was highly amplified leading to synergistic gains on bone mass. The mean and standard deviation (mean ± SD) of each bone parameter is reported.

	Combination Treatment		Short-Term			Long-Term		
			Vertebral TbN (1/mm)	Vertebral TbTh (mm)	Vertebral BV/TV	Vertebral TbN (1/mm)	Vertebral TbTh (mm)	Vertebral BV/TV
HET	0 mg/kg PAM	PBS	5.787 ± 0.300	0.040 ± 0.002	0.224 ± 0.017	5.545 ± 0.360	0.042 ± 0.002	0.235 ± 0.026
		SclAb	6.208 ± 0.420	0.046 ± 0.002	0.289 ± 0.032	6.273 ± 0.616	0.054 ± 0.003	0.339 ± 0.043
	0.3 mg/kg PAM	PBS	6.137 ± 0.536	0.043 ± 0.005	0.263 ± 0.050	6.002 ± 0.444	0.043 ± 0.003	0.257 ± 0.037
		SclAb	6.351 ± 0.303	0.044 ± 0.003	0.283 ± 0.024	6.706 ± 0.378	0.056 ± 0.003	0.378 ± 0.034
	0.625 mg/kg PAM	PBS	6.376 ± 0.569	0.039 ± 0.004	0.252 ± 0.045	6.300 ± 0.614	0.043 ± 0.004	0.275 ± 0.046
		SclAb	6.544 ± 0.423	0.045 ± 0.004	0.298 ± 0.043	7.028 ± 0.398	0.060 ± 0.006	0.421 ± 0.053
WT	0 mg/kg PAM	PBS	6.130 ± 0.271	0.044 ± 0.002	0.271 ± 0.019	6.233 ± 0.446	0.047 ± 0.004	0.293 ± 0.037
		SclAb	6.816 ± 0.376	0.053 ± 0.004	0.365 ± 0.040	6.860 ± 0.357	0.066 ± 0.008	0.452 ± 0.065
	0.3 mg/kg PAM	PBS	6.573 ± 0.459	0.045 ± 0.002	0.299 ± 0.034	6.487 ± 0.346	0.045 ± 0.002	0.291 ± 0.019
		SclAb	6.935 ± 0.519	0.055 ± 0.003	0.380 ± 0.041	6.917 ± 0.353	0.069 ± 0.007	0.478 ± 0.066
	0.625 mg/kg PAM	PBS	6.886 ± 0.363	0.043 ± 0.002	0.295 ± 0.021	6.838 ± 0.340	0.046 ± 0.003	0.315 ± 0.033
		SclAb	7.012 ± 0.167	0.055 ± 0.004	0.388 ± 0.029	7.091 ± 0.254	0.071 ± 0.007	0.504 ± 0.053

Table 3.5: *Effects on femoral mechanical and cortical properties from a single and concurrent cycles of combination therapy.* Femoral cortical analysis revealed SclAb influence on CTh varies in the presence of PAM after a single treatment cycle. Following subsequent cycles of combination therapy, CTh nearly doubled solely in response to SclAb. Functionally, multiple cycles PAM and SclAb led to an additive gains in femoral stiffness and ultimate load with progressive PAM doses. The mean and standard deviation (mean ± SD) of each bone parameter is reported.

	Combination Treatment		Short-Term	Long-Term				
			CTh (mm)	CTh (mm)	Stiffness (N/mm)	Ultimate Load (1/N)	Bending Moment (mm^4)	Cross-sectional Area (mm^2)
HET	0 mg/kg PAM	PBS	0.152 ± 0.016	0.191 ± 0.020	144.506 ± 30.164	23.056 ± 5.848	0.137 ± 0.023	1.798 ± 0.183
		SclAb	0.182 ± 0.008	0.228 ± 0.020	223.330 ± 40.350	33.668 ± 5.549	0.159 ± 0.029	1.919 ± 0.173
	0.3 mg/kg PAM	PBS	0.158 ± 0.020	0.193 ± 0.022	158.177 ± 42.292	23.454 ± 7.157	0.123 ± 0.039	1.694 ± 0.246
		SclAb	0.171 ± 0.017	0.233 ± 0.015	218.861 ± 37.982	35.421 ± 5.347	0.154 ± 0.016	1.895 ± 0.106
	0.625 mg/kg PAM	PBS	0.160 ± 0.017	0.189 ± 0.018	132.610 ± 7.296	23.139 ± 4.127	0.124 ± 0.029	1.719 ± 0.188
		SclAb	0.176 ± 0.012	0.232 ± 0.031	253.144 ± 54.500	37.049 ± 9.326	0.159 ± 0.040	1.942 ± 0.321
WT	0 mg/kg PAM	PBS	0.192 ± 0.022	0.226 ± 0.018	175.234 ± 30.630	29.775 ± 3.926	0.170 ± 0.040	1.996 ± 0.247
		SclAb	0.234 ± 0.009	0.277 ± 0.024	255.975 ± 39.086	47.690 ± 10.248	0.209 ± 0.063	2.261 ± 0.372
	0.3 mg/kg PAM	PBS	0.202 ± 0.021	0.220 ± 0.020	176.938 ± 28.273	31.590 ± 7.844	0.155 ± 0.023	1.949 ± 0.149
		SclAb	0.234 ± 0.009	0.277 ± 0.028	284.939 ± 60.078	48.121 ± 8.361	0.206 ± 0.039	2.209 ± 0.184
	0.625 mg/kg PAM	PBS	0.197 ± 0.014	0.222 ± 0.018	187.297 ± 38.584	31.235 ± 5.089	0.162 ± 0.028	1.967 ± 0.194
		SclAb	0.216 ± 0.028	0.277 ± 0.018	276.983 ± 73.111	47.638 ± 11.204	0.207 ± 0.033	2.230 ± 0.245

Table 3.6: *Effects on vertebral mechanical and cortical properties from a single and concurrent cycles of combination therapy.* Vertebral cortical analysis revealed that after a single cycle, SclAb triggered greater gains in CTh than when combined with PAM. Following two cycles, however, consistent gains in CTh were observed across all PAM dosages. Functionally, following a single combination cycle, PAM effect on trabecular preservation helped improve vertebral stiffness while SclAb amplified these effects. Significant improvements in ultimate load were solely attributed to SclAb. Both drugs improved ultimate load through an additive response, however, induced a synergistic effect on vertebral stiffness following multiple cycles of combination treatment. The mean and standard deviation (mean ± SD) of each bone parameter is reported.

	Combination Treatment		Short-Term			Long-Term		
			CTh (mm)	Stiffness (N/mm)	Ultimate Load (1/N)	CTh (mm)	Stiffness (N/mm)	Ultimate Load (1/N)
HET	0 mg/kg PAM	PBS	0.079 ± 0.006	31.193 ± 4.971	15.750 ± 7.562	0.078 ± 0.005	46.974 ± 20.181	22.696 ± 8.179
		SclAb	0.091 ± 0.005	42.387 ± 4.954	27.523 ± 8.172	0.091 ± 0.006	81.307 ± 15.531	61.764 ± 17.321
	0.3 mg/kg PAM	PBS	0.082 ± 0.011	49.112 ± 3.969	24.154 ± 8.213	0.081 ± 0.006	76.644 ± 15.154	31.330 ± 7.338
		SclAb	0.086 ± 0.007	61.102 ± 17.992	27.650 ± 6.055	0.090 ± 0.006	97.269 ± 13.733	56.919 ± 7.565
	0.625 mg/kg PAM	PBS	0.084 ± 0.012	54.149 ± 12.489	19.110 ± 7.156	0.083 ± 0.006	75.650 ± 8.031	31.257 ± 5.078
		SclAb	0.088 ± 0.007	68.106 ± 17.089	29.370 ± 4.233	0.093 ± 0.009	130.945 ± 25.920	69.416 ± 12.274
WT	0 mg/kg PAM	PBS	0.094 ± 0.002	44.237 ± 7.252	31.642 ± 10.346	0.088 ±0.008	95.297 ± 15.027	56.810 ± 14.635
		SclAb	0.100 ± 0.005	59.329 ± 11.964	48.157 ± 9.847	0.102 ± 0.008	136.688 ± 16.367	80.503 ± 9.545
	0.3 mg/kg PAM	PBS	0.096 ± 0.003	54.834 ± 8.050	28.608 ± 10.154	0.091 ± 0.005	93.523 ± 11.164	43.526 ± 4.540
		SclAb	0.102 ± 0.006	70.698 ± 17.131	46.238 ± 11.460	0.103 ± 0.010	151.576 ± 20.854	77.919 ± 17.028
	0.625 mg/kg PAM	PBS	0.098 ± 0.005	65.371 ± 7.165	29.423 ± 4.186	0.096 ± 0.006	119.729 ± 24.514	49.696 ± 11.258
		SclAb	0.102 ± 0.007	86.126 ± 19.143	47.382 ± 14.559	0.106 ± 0.008	156.340 ± 28.962	81.803 ± 12.247

Chapter 4 Conclusions

The purpose of these studies was to identify potential treatment plans that would provide the best clinical outcome to OI patients who require intervention during pregnancy or lactation and patients with severe OI. Based on the findings from these studies, the following conclusions, limitations and future studies are presented:

Bisphosphonate Treatment Intervention during Pregnancy and Lactation Leads to Temporal Inhibition of Lactation-Induced Bone Loss on the Maternal Skeleton

- Pregnancy-induced bone mass gains were solely attained through significant increase in Tb.N retention while lactation-induced bone loss were attributed to loss in both Tb.N and Tb.Th

- PAM intervention amplified pregnancy-induced bone mass gains and prevented lactation-induced bone loss when administered prior to conception (PC)

- Temporal effects from BP treatment during pregnancy and lactation showed that PAM was more effective in preserving maternal bone mass when treatment was administered at early stages of pregnancy (E15) or lactation (D1) and no preservation was observed when treated at late stages of lactation (D10 or D15)

- PAM amplified pregnancy-induced vertebral stiffness gains and induced a temporal conservation of femoral and vertebral stiffness when assessed following lactation

- No negative fetal or neonatal skeletal effects were observed in offspring from PAM treated dams despite preventing lactation-induced maternal bone loss
- PAM treatment had no effect on pregnancy-induced osteocyte osteolysis
- Greater osteoclast numbers were observed while osteoclast surface coverage remained unaffected with PAM treatment

Limitations

This study focused on assessing maternal skeletal effects following PAM administration and how maternal exposure may affect embryonic and neonatal bone development. In order to assess long-term history of BP therapy, a single PAM dosage was administered prior to conception, which does not reflect continuous BP accumulation in the maternal skeleton due to extensive treatment prior to conception in humans. However, we chose this approach because BPs would be embedded on the surface of the maternal skeleton rather than sequestered deep within the bone matrix, thus making BPs more readily available for offspring to be exposed to recycled BPs secondary to maternal bone resorption. A more representative approach to assess the effects from long-term exposure to BPs during pregnancy and lactation would be to pre-treat female dams with multiple cyclic BP treatments prior to conception. This would in turn achieve a more representative model of BP accumulation in maternal bone that can be recycled through maternal bone resorption and be a potential risk to fetal and neonatal bone development.

We only explored one PAM dose representative of a standard clinical dosage that has been used during pregnancy to create a snapshot of bone structure prior to changes in bone metabolism that are triggered by pregnancy and lactation-induced changes in calcium homeostasis. In addition, we

did not want over-saturate the system to induce any toxicity effects. However, exploring a wider range of dosages would fully assess the risk/benefit ratio of bone interventions during pregnancy and lactation. While studies have explored the effects of toxic dosages of BPs during pregnancy and lactation [79-82, 89, 113], better understanding of the risks of BPs can be derived from studies identifying the threshold of toxicity during pregnancy and lactation. Furthermore, treatment timing will also vary depending on fracture risk diagnosis during pregnancy and lactation to attain the best clinical outcome for both mother and offspring. For example, if treatment intervention is needed during pregnancy, it might be best to treat with BPs prior to fetal ossification to avoid possible BP accumulation in the embryo. On the other hand, if intervention is needed towards the end of lactation, a best clinical practice might be to stop lactation since studies have shown post-lactation bone recovery [132, 133, 202, 203] and our study showed no differences between untreated and treated dams by D10 and D15 of lactation.

It is now well established that BPs have a long half-life and remain embedded in the bone matrix until liberated during osteoclastic resorption [18, 69]. Thus, if significant levels of BPs are incorporated into the fetal and neonatal skeleton during pregnancy and/or lactation, BP effects might not be observed until later stages of rapid growth or adulthood when BPs are revealed through bone remodeling. Therefore, although no effects were observed in this study on weaned pups, BP exposure risk should not be disregarded and further post-lactation assessment of neonatal growth should be considered. In this study, our goal was to analyze the immediate effects in bone morphology resulting from potential PAM exposure during skeleton development. However, a more direct approach to evaluate the risk of BP exposure during neonatal bone development, as well as the role of osteoclasts during early stages of mineralization would be to directly treat neonatal mice immediately following birth to contrast direct vs. indirect exposure to the drug.

Despite inhibiting lactation-induced maternal bone mass, no effects were observed in fetal and neonatal bone development. However, we did not analyze calcium levels in mothers or their offspring to verify that PAM did not induce hyper/hypocalcemia. To evaluate if direct disruption of maternal calcium homeostasis from PAM-induced anti-resorptive effects prevented offspring from receiving the necessary calcium supply, analyzing Ca+ levels in breast milk could help address risk of hypocalcemia in neonates. Additionally, evaluating the quality of bone mineralization could also help address the difference in calcium content between offspring from treated and untreated dams. Due to limited microCT resolution and high porosity in neonatal skeleton at day 1, we were only able to quantify BMD, but techniques like scanning electron microscope energy dispersive analysis of X-rays (SEM-EDAX) could be used to evaluate the differences in elemental composition between neonatal bone surfaces from treated and untreated mothers. Furthermore, collecting serum or urine samples from pups to perform analysis on osteoclast and osteoblast activity could further assess the indirect impact that BP inhibition of maternal bone loss has on neonatal skeleton development.

Aside from changes in maternal bone metabolism during pregnancy and lactation, several hormones like parathyroid hormone related peptide (PTHrP), calcitonin, 1,25 dihydroxy-vitamin D, estradiol, prolactin and several others have been shown to play a crucial role in elevating intestinal calcium absorption during pregnancy and maintaining calcium homeostasis during lactation [158, 204]. However, the mechanisms and role which these hormones play to drive bone loss during lactation are only partly understood. Vitamin D has been shown to play a role in upregulating maternal calcium absorption, while PTHrP, along with local changes at the bone level involving receptor activator of nuclear factor kappa B ligand and osteoprotegerin, have been implicated in increasing bone resorption, allowing maternal calcium stores to supply calcium to

the fetus [205, 206]. Serum calcitonin levels are increased during pregnancy and potentially play a role in protecting the maternal skeleton against excessive resorption during lactation [158]. Pregnancy also involves changes in estradiol and prolactin, which are produced by the placenta and exhibit an effect on bone metabolism. Studies have shown that estrogen along with PTHrP help regulate bone resorption during pregnancy and lactation [207, 208]. Animal studies have shown that a surge in prolactin and PTHrP in combination with a decline in estradiol and progesterone stimulate lactation events [128, 208, 209]. Thus potential intestinal calcium absorption and hormonal changes in the presence of an anti-resorptive like PAM need to be addressed to determine which calcium pathway is affected by the anti-resorptive effects of BPs.

Future Directions

Before considering translation to humans, the limitations stated above need to be addressed, especially when assessing the safety of BP exposure during fetal and neonatal bone development. Although we did not observe any differences in body mass, bone morphology and osteoclast activity between offspring from untreated and treated dams, results from PAM exposure were evaluated at birth and weaned date. However, since our PAM dosage was enough to cross the placenta and diffuse into the breast milk, localized BP can still be recycled during rapid growth and affect the offspring at later stages in life. Therefore, bone morphology and osteoclast activity should be evaluated post-rapid growth or later in adulthood after onset of osteoclastogenesis to fully address the effects of BPs. On the maternal side, we can also assess post-lactation effects from PAM exposure during pregnancy and lactation. While studies have already shown that inhibition of lactation-induced bone loss does not affect bone mass gains post-lactation, we can address if a single PAM treatment is enough to retain trabecular structure post-lactation. In

addition, running this study in parallel with WT dams will allow us to understand the differences in lactation-induced bone loss and bone recovery when an underlying bone disease is present.

Unlike BPs, anabolic therapies have not shown to have a long-half life, however the effects of anabolic therapy during pregnancy and lactation have not been studied. Since SclAb has shown promising results in preclinical [41, 44-53] and clinical studies [58, 59], repeating the presented study with an anabolic treatment would allow us to further understand bone and calcium metabolism during these reproductive periods. More importantly, because SclAb has a large molecular weight (24kDa), there is significantly less exposure risk to the offspring. Given that pregnancy and lactation induce a significant amount of bone loss, a single intervention with SclAb might be enough to recover bone loss if the BP treatment window is missed since this treatment window could potentially be expanded with an anabolic therapy. Furthermore, we hypothesize that the offspring from this type of study would remain unaffected since maternal calcium homeostasis will not be directly affected through anabolic targeting of osteoblast-mediated bone formation instead of osteoclast activity. Thus, maternal bone formation might be triggered, ensuring that enough calcium is being transferred to the developing fetal and neonatal skeleton. Additionally, it would be interesting to see if the same degree of temporal bone loss is expressed with anabolic therapy. While in the present study we showed that inhibition of lactation-induced bone loss still provided the necessary supply of calcium to the developing offspring and since some studies have shown that calcium supplementation does not prevent lactation-induced bone loss [136, 154, 210-215], it would also be interesting to evaluate if additional bone formation can be triggered with an anabolic therapy in combination with a calcium enriched diet to reduce the amount of maternal bone loss during lactation.

Another important study that can be derived from the present results is to focus entirely in the pathways that maintain calcium homeostasis during physiological changes in the maternal skeleton. This can be achieved by designing a study that combines both BPs and maternal diet (vitamin D and calcium deficiencies) to further understand what pathway drives calcium homeostasis during this period of high calcium demand. The main groups in the study will be untreated dams in a calcium deficient diet, and BP treated dams in a calcium deficient diet, as it would allow us to understand the role intestinal absorption has in calcium homeostasis compared to bone resorption.

Low Dose of Bisphosphonate Enhances Sclerostin Antibody-Induced Trabecular Bone Mass Gains in Brtl/+ Osteogenesis Imperfecta Mouse Model

- PAM induced a sclerotic metaphyseal band confirming BPs retention of primary trabeculae
- Lack of long-term antiresorptive effects were observed in trabecular bone following our low dose treatment of PAM monotherapy
- A significant dose dependent preservation of Tb.N was induced by PAM monotherapy
- SclAb monotherapy led to gains in both Tb.N and Tb.Th
- Lack of a saturation response from combination therapy was confirmed since both interventions stimulated gains in bone mass through independent means
- Gains in cortical thickness were attributed to SclAb alone and progressive gains were observed following two cycles of SclAb monotherapy
- Immediate effects of PAM and SclAb led to cumulative gains in trabecular bone mass triggered which were attributed to PAM retention of trabecular number and SclAb-induced trabecular thickening

- Following two cycles of combination therapy a cumulative increase in bone mass was observed in long bones while a synergistic effect was observed in the vertebral body
- SclAb was the sole contributor of increased stiffness and ultimate load in the femur
- The whole-bone mechanical properties of the vertebra showed that SclAb contributed to improvements in ultimate load while synergistic gains in stiffness were observed after two cycles of combination treatment

Limitations

This study has several limitations. Because OI is a disease of many mutations, the results of this study may not extend to all types of OI. Although the Brtl/+ mouse fragility phenotype is of moderate severity, it does not suffer spontaneous fractures. In order to verify that our PAM and SclAb combination therapy will be effective in severe cases, we need to assess the effects in models like the osteogenesis imperfecta mouse model (oim) strain or Col1a1+/^{jrt} mouse model of severe dominant OI. Studies have shown that the oim model has a mutation in the pro-alpha2 chain of type I collagen resulting in acute osteopenia, bone deformities and spontaneous fractures [216, 217]. A more severe model is the Col1a1+/^{jrt} model, which has shown signs of high bone turnover, spontaneous fractures, and deformities [218, 219]. Due to their more severe phenotype, these models can help confirm the clinical importance of treating severe cases of OI with an antiresorptive therapy in combination with an anabolic therapy.

In many of our outcomes, treatment with our lowest PAM dose alone improved bone mass to WT control levels, effectively rescuing the phenotype, while our higher PAM dose induced gains in bone mass greater than WT baseline. Although our goal was not to directly titrate our treatment outcomes to match untreated WT levels, we believe that further adjustment of treatment doses

and/or duration will vary depending on clinical severity to best represent a "rescue" response. Furthermore, when dealing with combination treatment studies, the order, timing between treatments and doses are highly dependent on the clinical outcome one is trying to achieve.

Future Directions

BP mechanism of action when given after closure of the growth plate during skeletal maturity will not yield the same findings as observed in this study because turnover of primary spongiosa will be nonexistent. In other words, the synergistic benefits observed in this growing model might be different in adult mice. However, addressing these effects in an older animal model will be beneficial, as cases of severe osteoporosis in post-menopausal women with OI have been reported [220]. Furthermore, in an adult model, it may be more beneficial to build bone with an anabolic therapy and then preserve it with an anti-resorptive.

In order to determine the correct number of cycles and timing between cycles for minimum drug treatment but equivalent results, a cessation study can be performed to evaluate the long-term effects from a single combination cycle. This will allow us to better control the effects of both drugs combined in order to avoid a plateau effect. Additionally, understanding the cessation effect from PAM and SclAb combination therapy will also help us maintain the treated bone structure to WT levels.

Given the lack of treatment options for patients with severe OI and the lack of knowledge regarding the effects of BPs during pregnancy and lactation on both the maternal and fetal/neonatal skeleton; this thesis provides valuable insight that will help promote strategic and thought out therapeutic interventions for challenging clinical scenarios. Rather than developing new pharmacological drugs to achieve a "one treatment fits all" solution, novel approaches to therapeutic can be designed

using current and promising antiresorptive and anabolic therapies. By understanding the changes in bone metabolism of the clinical conditions under investigation and by combining this knowledge with our understanding of the targeted pathways of available and emerging pharmaceuticals, we can strategically and systematically optimize bone therapeutics so that the best clinical outcome can be achieved.

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